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**(54) PROCESS FOR PRODUCING TRANSGLUTAMINASE**

(57) The present invention relates to a process for secretory production of a foreign protein, in particular, transglutaminase by a coryneform bacterium.

According to the present invention, a process is provided for the secretory production of a foreign protein, in particular, transglutaminase, by making a coryneform bacterium to produce an industrially useful foreign protein, in particular, transglutaminase and efficiently release the product extracellularly (i.e., secretory production).

An intended foreign protein, in particular, transglutaminase, is produced by using an expression con-

struct wherein the gene sequence of the intended foreign protein containing the pro-structure part, in particular, pro-transglutaminase gene sequence, is ligated to the downstream of a sequence encoding the signal peptide region from a coryneform bacterium, introducing this expressional genetic construct into a coryneform bacterium, culturing the thus transformed coryneform bacterium, and treating the extracellularly released protein with a protease, etc. to cleave and eliminate the pro-part.

EP 1 219 713 A1

## Description

## Background of the invention

5 [0001] The present invention relates to a process of producing a heterologous protein, especially transglutaminase, by secretory production. The heterologous proteins produced by the method include industrially useful enzymes, physiologically active proteins and others. Transglutaminase has been widely used for food processing, the manufacture of pharmaceuticals and the like.

10 [0002] A number of processes for the secretory production of heterologous proteins have been previously reported including those as described in the review on the secretory production of a heterologous protein by a bacterium belonging to the genus *Bacillus* [Microbial. Rev., 57, 109-137 (1993)], the review on the secretory production of a heterologous protein by methylotrophic yeast *Pichia pastoris* [Biotechnol., 11, 905-910 (1993)] and the report on the industrial production of heterologous proteins by the mould belonging to the genus *Aspergillus* [Biotechnol., 6, 1419-1422 (1988); Biotechnol., 9, 976-981 (1991)].

15 [0003] The transglutaminase produced by the secretory production according to one embodiment of the present invention is an enzyme which catalyzes acyltransfer reaction of  $\gamma$ -carboxylamide groups in the peptide chain of the protein. When the enzyme is reacted with a protein, the formation of the cross-linkage  $\epsilon$ -( $\gamma$ -Glu)-Lys and the replacement of Gln with Glu by deamidation can be occurred. Transglutaminase has been used to manufacture gelled food products such as jelly, yogurt, cheese or gelled cosmetics and others, and to improve the quality of meat, etc (Japanese publication of examined application No. 1-50382). Moreover transglutaminase is an enzyme having industrially high usefulness in that it has been used to manufacture materials for thermostable microcapsules, carriers for immobilized enzymes etc.

20 [0004] Transglutaminases derived from animals and from microorganisms (microbial transglutaminase: referred to as 'MTG' hereinafter) have been previously known. The former is the calcium ion-dependent enzyme which is distributed in animal organs, skin, blood, etc. The examples include guinea pig hepatic transglutaminase (K. Ikura et al. Biochemistry 27, 2898 (1988)), human epidermal keratinocyte transglutaminase (M. A. Phillips et al. Proc. Natl. Acad. Sci. USA 87, 9333 (1990)), human blood coagulation factor XIII (A. Ichinose et al. Biochemistry 25, 6900 (1990)) and others.

25 [0005] For the latter, calcium-independent transglutaminases have been discovered from bacteria belonging to the *Streptovorticillium* genus, which include, for example, *Streptovorticillium griseocarneum* IFO 12776, *Streptovorticillium cinnamoneum* sub sp. *cinnamoneum* (hereinafter it can be abbreviated as *S. cinnamoneum*) IFO 12852, *Streptovorticillium mobaraense* (hereinafter it may be abbreviated as *S. mobaraense*) IFO 13819 and others (Publication of unexamined Japanese patent application (JP-Kokai) No. 64-27471). The peptide mapping and the structural analysis of the genes revealed that the primary structure of the transglutaminase produced by these microorganisms shared no homology with transglutaminases from animals (European Patent application No. 0 481 504 A1).

30 [0006] Because microorganism-derived transglutaminase (MTG) is produced through the purification from the cultures of microorganisms such as described above, there have been problems in terms of the amount and the efficiency and the like. The production of transglutaminase using genetically engineered procedure has been also attempted. Transglutaminase proteins and the genes thereof have been reported in, for example, Biosci. Biotechnol. Biochem., 58, 82-87 (1994), Biosci. Biotechnol. Biochem., 58, 88-92 (1994), Biochimie, 80, 313-319 (1998), Eur. J. Biochem., 257, 570-576 (1998), WO 96/06931, WO 96/22366, etc, which report the expression and production of transglutaminase in host-vector systems such as *Streptomyces lividans*, *Aspergillus oryzae* and *Escherichia coli*. In addition to these information, a process wherein transglutaminase is produced by secretory production in microorganisms such as *E. coli* and yeast (JP-Kokai No. 5-199883) and the method has been reported wherein MTG having activities is produced by expressing MTG as an inactive fused protein in an inclusion body within *E. coli* and subsequently solubilizing the inclusion body using protein-denaturing agents, and then, reconstituting it through the removal of the denaturing agents (JP-Kokai No. 6-30771). However, the problem has been noted that the expression level is significantly low in the secretory production by microorganisms such as *E. coli* or yeast.

35 [0007] On the other hand, there are examples of previous studies for the efficient secretory production of heterologous proteins using a coryneform bacterium include the secretion of nucleases and lipases [US4965197, J. Bacteriol., 174, 1854-1861 (1992)] and the secretion of proteases such as subtilisin [Appl. Environ. Microbiol., 61, 1610-1613 (1995)] by *Corynebacterium glutamicum* (hereinafter it may be abbreviated as *C. glutamicum*), a study on the secretion of cell surface proteins of a coryneform bacterium [International patent application published in Japan No. Hei 6-502548], the secretion of fibronectin-binding protein using this study [Appl. Environ. Microbiol., 63, 4392-4400 (1997)], a report wherein the secretion of proteins was enhanced using a mutated secretory machinery [JP-Kokai No. 11-169182], etc., but there has been a limited number of reports on limited proteins. In light of the accumulated amount of proteins, Appl. Environ. Microbiol., 61, 1610-1613 (1995) describes that about 2.5 mg/ml of protein was accumulated by expressing the alkaline protease gene from *Dichelobacter nodosus* within *C. glutamicum* using a promoter of subtilisin gene (*aprE*) from *Bacillus subtilis*, ribosome binding site and the sequence of a signal peptide, but US4965197, JP-Kokai No.

6-502548 or JP-Kokai No. 11-169182 do not specifically describe the values of the amount of the proteins secreted and accumulated. Furthermore, in the case of the fibronectin-binding protein [Appl. Environ. Microbiol., 63, 4392-4400 (1997)], only the secretory accumulation of the protein of about 2.5 µg/L is confirmed. Thus, there has been no reports that heterologous proteins could be efficiently accumulated in the medium at a practical level.

[0008] Additionally a genetic engineering technology for a coryneform bacterium has been developed in the system using plasmid and phage, such as the establishment of the transformation by protoplast [J. Bacteriol., 159, 306-311 (1984); J. Bacteriol., 161, 463-467(1985)], the development of a various type of vectors [Agric. Biol. Chem., 48, 2901-2903(1984); J. Bacteriol., 159, 306-311(1984); J. Gen. Microbiol., 130, 2237-2246(1984); Gene, 47, 301-306 (1986); Appl. Microbiol. Biotechnol., 31, 65-69(1989)], the development of the regulation method of gene expression [Bio/Technology, 6, 428-430(1988)] and the development of cosmid [Gene, 39, 281-286(1985)]. Moreover there are reports on the cloning of genes from a coryneform bacterium [Nucleic Acids Res., 14, 10113-1011(1986); J. Bacteriol., 167, 695-702(1986); Nucleic Acids Res., 15, 10598(1987); Nucleic Acids Res., 15, 3922(1987); Nucleic Acids Res., 16, 9859(1988); Agric. Biol. Chem., 52, 525-531(1988); Mol. Microbiol., 2, 63-72(1988); Mol. Gen. Genet., 218, 330-339 (1989); Gene, 77, 237-251(1989)].

[0009] Further a transposable element derived from a coryneform bacterium has also been reported [WO93/18151; EP0445385; JP-Kokai No. 6-46867; Mol. Microbiol., 11, 739-746(1994); Mol. Microbiol., 14, 571-581(1994); Mol. Gen. Genet., 245, 397-405(1994); FEMS Microbiol. Lett., 126, 1-6(1995); JP-Kokai No. 7-107976].

[0010] The transposable element means a DNA fragment which can be transposed on the chromosome and is known to be present in a wide range of organisms ranging from prokaryotes to eukaryotes. Transposons using transposable elements have been developed [WO93/18151; JP-Kokai No. 7-107976; Mol. Gen. Genet., 245, 397-405(1994); JP-Kokai No. 9-70291] and a heterologous gene has become to be able to be expressed using a transposon.

#### Summary of the Invention

[0011] An object of the invention is to provide a process for the production of a heterologous protein, in particular transglutaminase by making a coryneform bacterium to produce an industrially useful heterologous protein, in particular, transglutaminase and efficiently releasing the product extracellularly (i.e., secretory production).

[0012] The inventors have found a process for the effective secretory production of industrially useful heterologous protein, in particular transglutaminase, taking notice of the fact that a pro-part as well as a signal peptide in a secretory protein of actinomycetes and the like play an important role in the secretion process.

[0013] Therefore, the present invention is a process for producing a heterologous secretory protein characterized in that a fusion protein is produced and secreted (secreto-produced) in a coryneform bacterium, wherein the heterologous secretory protein contains its pro-part ligated to the downstream of the signal peptide domain from a coryneform bacterium and then the pro-part is cleaved and eliminated.

[0014] More specifically, the invention is a process to obtain a large amount of an intended heterologous protein, in particular transglutaminase by transferring a genetic expression construct into a coryneform bacterium, wherein a gene sequence of an intended protein containing a pro-structure part, in particular, the pro-transglutaminase gene sequence, which is ligated to the downstream of a sequence encoding the signal peptide domain from a coryneform bacterium, especially the signal peptide domain of a cell surface protein, culturing the thus transformed coryneform bacterium, efficiently releasing the resulting protein extracellularly and treating the protein which is released outside the cells with a protease, etc. to cleave the pro-part.

[0015] The invention is also a process to obtain transglutaminase in which the pro-structure part of protransglutaminase is cleaved, wherein an expression construct for protease and the like are also generated in the same fashion as with the genetic construct for transglutaminase, introducing it into a coryneform bacterium together with the expression construct containing the protransglutaminase gene and culturing thus transformed coryneform bacterium, or introducing the expression construct for protease and the like into another coryneform bacterium and culturing the transformed coryneform bacterium together with the pro-transglutaminase gene-introduced bacterium, to express and secrete the protransglutaminase and the protease.

[0016] As used herein, "the secretion" of a protein or peptide refers to the transportation of the protein or peptide molecule outside the bacterium cell (extracellular transportation) including the case where the protein or peptide molecule exist finally in completely free form in the medium as well as the case where only the part of the protein or peptide molecule is present outside the cell and the case where they are located on the surface of the cell.

#### Best Mode for Carrying Out the Invention

[0017] According to the process of the invention, a coryneform bacterium is used as a host vector system, and a large amount of transglutaminase, from which the pro-structure part is removed, may be obtained by generating an expression construct wherein a transglutaminase gene containing a pro-structure part in secretory form is ligated to

the downstream of the signal peptide of cell surface protein from coryneform bacterium, introducing and expressing the construct into a coryneform bacterium, and treating the pro-structure part of the protransglutaminase secreted extracellularly with a protease and the like to cleave the pro-structure part.

[0018] According to the process of the present invention, a transglutaminase in which the pro-part is cleaved can be directly obtained extracellularly by generating a genetic expression construct for a protease and the like in a similar manner as for the protransglutaminase genetic construct, introducing and expressing it into the coryneform bacterium together with protransglutaminase genetic construct and culturing the thus transformed coryneform bacterium, or introducing the genetic expression construct for the protease and the like into another coryneform bacterium and culturing the thus transformed coryneform bacterium together with the bacterium where protransglutaminase has been introduced, and thus making the bacterium to express and secrete the protransglutaminase and the protease.

[0019] A secretory protein has been generally known to be translated as a prepeptide or prepropeptide and thereafter to be formed into a mature protein. That is to say, in general, it has been known that it is translated as a peptide or prepropeptide, then the signal peptide ("a pre-part") is cleaved, thereby it is converted into a mature peptide or propeptide by further cleaving of the pro-part with a protease. As used herein, "a signal sequence" refers to the sequence which is located at the N-terminal of a secretory protein precursor and which is not present in a naturally occurring mature protein, and "a signal peptide" refers to the peptide which is cleaved from such a protein precursor. Generally, a signal sequence is cleaved coupling the extracellular secretion by a protease (generally referred to signalpeptidase). Although such a signal peptide shares certain common features in the sequence over species, a signal peptide which has secretory function in one species does not necessarily have the same secretory function in another species.

[0020] As used herein, a protein which contains both a signal peptide and a pro-part, that is, a primary translation product can be referred to "a preproprotein", and a protein which does not contain a signal peptide but does contain a pro-part can be referred to "a proprotein". A pro-part of a proprotein can be referred to "a pro-structure part" or "a pro-structure". "A pro-structure part/pro-structure" of a protein can be herein interchangeably used with "a pro-part" of a protein. A signal peptide in a preproprotein or proprotein may be derived from a different protein or be a signal peptide naturally occurring in the intended protein and it is preferably derived from a secretory protein of the host to be used. Alternatively, it may be modified to have the optimum codon depending on the codon usage of the host to be used. Moreover, the signal peptide that can be used for the purpose of the invention may contain a part of the N-terminal amino acid sequence of a naturally occurring mature protein from which the signal peptide is derived. A preproprotein can be especially called "a heterologously fused preproprotein" when the signal peptide is derived from the different protein. For example, when a protein is transglutaminase, they are referred to "preprotransglutaminase", "protransglutaminase" and "heterologously fused preprotransglutaminase", respectively. A protein in which the pro-part is cleaved is referred to a protein wherein at least one or more amino acid that constitute the pro-part is removed by cleaving the peptide bond, including a protein having identical N-terminal amino acid with the naturally occurring protein and also includes a protein having one or more extra amino acids at the N-terminal deriving from the pro-part compared to the naturally occurring protein, and a protein having shorter amino acids sequence than that of a naturally occurring mature protein, provided that the protein has an activity of the intended protein.

[0021] As is described as the prior art, a limited number of reports have been shown where the extracellular secretory production of a heterologous protein has been achieved using coryneform bacterium and the secretory production process have not been technically completed. Also, it has not been known that a coryneform bacterium extracellularly secretes a protein such as a protease by itself. The known examples are endogenous DNase [US4965197] and the facts that the cell surface protein used in the present invention falls off from the cell surface to be found outside the cell [JP-Kokai No. 6-502548]. However, any signal peptide that involves in the secretion of a protein of coryneform bacterium has not previously known except for the cell surface proteins. The only known cell surface proteins from coryneform bacterium, to date, are Genes for PS1 and PS2, the cell surface proteins of *Corynebacterium glutamicum* [JP-Kokai No. 6-502548], and the gene for SlpA, the cell surface protein of *Corynebacterium ammoniagenes* (which may be abbreviated as *C. ammoniagenes* hereinafter) [JP-Kokai No. 10-108675]. Among these proteins, PS1 and SlpA share some homology (about 30%), but almost no homology was found among others, and furthermore there found no homology in the signal sequence domain between each other. As the examples of signal sequences, the signal sequences of PS1 and PS2 from *Corynebacterium glutamicum* are shown in SEQ ID NO: 29 and SEQ ID NO: 1, and the signal sequence of SlpA from *Corynebacterium ammoniagenes* is shown in SEQ ID NO: 2.

[0022] Therefore, the inventors cloned the gene for PS2 protein from *C. glutamicum* (formerly, *Brevibacterium lactofermentum*) ATCC13869 strain and determined the sequence. It was found that there were no differences in the signal sequence domain from the known sequence from *C. glutamicum*, but that there were two different amino acids in the sequence up to the N-terminal thirty eighth amino acid residue of the mature cell surface protein (Asn for Thr residue at position 40 and Glu for Gly residue at position 55 in the amino acid sequence as, is shown in SEQ ID NO: 7). The nucleotide sequence encoding for sixty eight residues comprising thirty amino acid residues of the signal peptide and thirty eight amino acid residues from the N-terminal of the mature cell surface protein and its 5'-upstream region containing a promoter domain is shown in SEQ ID NO: 6 and the amino acid sequence is shown in SEQ ID NO: 7.



[0023] Then, the inventor examined the secretion of a heterologous protein using the region containing the promoter domain or the signal peptide domain of the cell surface protein in order to determine whether the extracellular secretory production of a large amount of the heterologous protein can be achieved in a coryneform bacterium.

[0024] Since the transglutaminase gene from actinomycetes has a high GC content and the gene from coryneform bacterium has a close GC content to the gene from actinomycetes and also they have closely similar codon usage, there is an advantage that the gene from actinomycetes can be directly used. Therefore, the inventor investigated whether a transglutaminase gene from actinomycetes can be directly used and found that the signal peptide of transglutaminase from actinomycetes did not successfully function in a coryneform bacterium. However, it is revealed that the transglutaminase gene encoding the mature protein containing the pro-structure part from actinomycetes fused with the signal peptide of the cell surface protein from a coryneform bacterium effectively functioned without any modification and was efficiently secreted outside the cell as proprotein containing the pro-structure part. When the gene for transglutaminase with the pro-structure part which additionally comprises thirty amino acid residues from the cell surface protein and thirty eight amino acid residues from the N-terminal domain of the mature cell surface protein, i. e., the gene for transglutaminase fused with the N-terminal domain of the mature cell surface protein, was used, the efficiency of the extracellular secretion of transglutaminase was further increased.

[0025] As used herein, a coryneform bacterium is an aerobic Gram-positive bacillus, which includes bacteria which was previously classified as Brevibacterium but currently unified as Corynebacterium (Int. J. Syst. Bacteriol., 41, 255 (1981)) including Brevibacterium which is closely related to Corynebacterium. An advantage in using Corynebacterium is that it inherently secretes extremely less proteins outside the cell compared to molds, yeasts or bacteria belonging to Bacillus which have been previously suitable to effect the secretion of a heterologous protein, which allow the purification process of the product to be simplified and shortened when the secretory production of a heterologous protein is conducted, and that it is excellent in terms of its medium cost, the culturing procedure and the yield, since it grows well on a simple culture medium such as those composed of ammonia, inorganic salts and so on.

[0026] Examples of Corynebacterium which can be used as a host bacterium in the present invention include wild type strains including Brevibacterium saccharolyticum ATCC14066, Brevibacterium immariophilum ATCC14068, Brevibacterium lactofermentum (Corynebacterium glutamicum) ATCC13869, Brevibacterium roseum ATCC13825, Brevibacterium flavum (Corynebacterium glutamicum) ATCC14067, Corynebacterium acetoacidophilum ATCC13870, Corynebacterium glutamicum ATCC13032, Corynebacterium lilium (Corynebacterium glutamicum) ATCC15990, Brevibacterium ammoniagenes (Corynebacterium ammoniagenes) ATCC6871, and mutant strains derived from these wild type strains, for example, mutant strains defective in the ability to produce glutamate, mutant strains for amino acids production such as lysine and the like, and mutant strains modified to produce other substances such as nucleic acids, for example, inosine.

[0027] The genetic construct which can be used in the present invention generally includes a promoter, a sequence encoding a proper signal peptide and a nucleic acid fraction encoding an intended protein, and a regulatory sequence (a operator or terminator, etc.) necessary to express the gene for the intended protein in a coryneform bacterium, at a proper position such that they can function. The intended protein may have a pro-structure part at the N-terminal. Vectors which can be used for this construct are not particularly limited and include any one which can function in a coryneform bacterium, and they may be those which autonomously multiply such as plasmids or vectors which are integrated into the chromosome of the bacterium. Plasmids derived from coryneform bacteria are particularly preferable. These include, for example, pHM1519 (Agric. Biol. Chem., 48, 2901-2903(1984)), pAM330 (Agric. Biol. Chem., 48, 2901-2903(1984)), and plasmids obtained by modifying them which possess drug-resistant genes. Artificial transposons and the like can be also used. When a transposon is used, the intended gene is introduced in the chromosome through homologous recombination or by its own transposing ability.

[0028] Promoters which can be used in the invention are not particularly limited. Any promoter which can function in the cell of a coryneform bacterium can be generally used. It may be also a promoter derived from a different species, for example, a promoter derived from E. coli, such as tac promoter, etc. Among these promoters, a potent promoter is more preferably, such as tac promoter, etc. Examples of promoters derived from a coryneform bacterium include promoters for the genes of cell surface proteins PS1, PS2 and SlpA, promoters for the genes in biosynthetic systems of different amino acids, for example, glutamate dehydrogenase gene in the glutamic acid biosynthetic system, glutamine synthetase gene in the glutamine synthetic system, aspartokinase gene in the lysine biosynthetic system, homoserine dehydrogenase gene in the threonine biosynthetic system, acetohydroxylate synthase gene in the isoleucine and valine biosynthetic system, 2-isopropylmalate synthase gene, glutamate kinase gene in the proline and arginine synthetic system, phosphoribosyl-ATP pyrophosphorylase gene in the histidine biosynthetic synthesis, deoxyarabinohepturonic acid phosphate(DAHP) synthase gene in the aromatic amino acid biosynthetic system such as tryptophan, tyrosine and phenylalanine, etc., phosphoribosylpyrophosphate (PRPP) amidotransferase gene, inosinate dehydrogenase gene and guanylate synthase gene in the nucleic acid biosynthetic system such as inosinate and guanylate.

[0029] The signal peptide which is used in the present invention is the signal peptide of a secretory protein from the host, Coryneform bacterium, and preferably it is the signal peptide of a cell surface protein from a Coryneform bacterium.

Cell surface proteins include PS1 and PS2 derived from *C. glutamicum* (JP-Kokai No. 6-502548), and SlpA derived from *C. Ammoniagenes* (JP-Kokai No. 10-108675). The amino acid sequence of PS1 is shown in SEQ ID NO:29, the amino acid sequence of PS2 in SEQ ID NO:1 and the amino acid sequence of SlpA in SEQ ID NO:2. Additionally, it is reported that DNase from a coryneform bacterium also has a signal peptide, as described in US Patent No. 4965197, which can be used in the present invention.

[0030] To the signal peptide, a portion of N-terminal amino acid sequence of the secretory protein from which the signal peptide derives may be connected. The signal sequence is cleaved by a signalpeptidase during the translated product is secreted extracellularly. In addition, the gene encoding the signal peptide can be used either in native form or in modified form to contain the optimum codons depending on the codon usage in the host to be used.

[0031] When these signal peptides are used, the genes encoding for intended proteins are ligated to the 3'-terminal of the genes encoding the signal peptides and are located such that they are subjected to the regulation of expressions by the promoters described above.

[0032] The useful proteins which can be secreto-produced according to the present invention essentially includes, but are not limited to, all of the secretory proteins derived from animals and plants and microorganisms. For example, proteins such as protease, aminopeptidase, carboxypeptidase, collagenase and chitinase can be secreto-produced according to the present invention. Proteins which are prepared by the secretory production according to the present invention are preferably naturally occurring secretory proteins, more preferably proteins having additional pro-structure parts. Transglutaminase is particularly preferred as a useful protein prepared by the secretory production according to the present invention. As transglutaminase genes, for example, genes for secretory transglutaminase derived from actinomycetes, for example, *S. mobaraense* IFO 13819, *S. cinnamoneum* IFO 12852, *Streptovorticillium griseocarcineum* IFO 12776, *Streptomyces lydicus* [WO9606931], etc. and molds such as *Oomyceted* [WO9622366], etc can be used for the purpose of the present invention. The genes encoding these proteins can be modified depending on the type of the host to be used and in order to achieve the desired activity, and comprise the addition, deletion, replacement of one or more amino acid residues and optionally may be converted into the optimum codon depending on the frequency of codon usage in the host.

[0033] When the protein prepared by the secretory production according to the present invention is the protein naturally expressed as a prepropeptide, the gene fragment encoding the proprotein containing the pro-structure part (pro-part) is preferably used. As examples of the sequences of the pro-parts, the sequences of the pro-structure parts of transglutaminases derived from actinomycetes are shown in SEQ ID NO:3 (derived from *S. mobaraense*) and SEQ ID NO:4 (derived from *S. cinnamoneum*). The pro-part of the protein may be cleaved by appropriate means, for example by proteases. Aminopeptidase, endopeptidase which cleaves it at a proper position, or more specific protease can be used. Preferably, the proteases which cleave the protein such that the resulting protein has an equivalent to or more activity than that of the naturally occurring protein. Alternately the gene sequence encoding the intended protein or the pro-structure part of the intended protein can be also modified and designed to express the protein having the recognition site for protease specific to the desired location. General molecular biotechnological procedures including such modification techniques, gene cloning techniques and detection techniques for the produced proteins are well known to those skilled in the art and reference can be made to Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, *DNA Cloning: A Practical Approach*, Volumes I and (D. N. Glover ed. 1985), F.M. Ausubel et al.(eds), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994), *PCR Technology: Principles and Application for DNA Amplification*, H. Erlich, ed., Stockton Press and etc.

[0034] Examples of pro-structure parts having the modified pro-structure parts which are shown in SEQ ID NO:3 and SEQ ID NO:4 includes the modified pro-structure parts as described in SEQ ID NO:30 to SEQ ID NO:38.

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Sequence list free texts

SEQ ID NO:30 to SEQ ID NO:37: the modified pro-structure part of transglutaminase from *S. mobaraense*

SEQ ID NO:38: the chimera of transglutaminase pro-structure parts of *S. mobaraense* and *S. cinnamoneum*

These modified pro-structure parts have the following features:

SEQ ID NO:30 = AP at the C-terminal of the pro-structure part from *S. mobaraense* (45 amino acid residues) is deleted;

SEQ ID NO:31 = FRAP at the C-terminal of the pro-structure part from *S. mobaraense* (45 amino acid residues) is deleted;

SEQ ID NO:32 = D at the N-terminal of the pro-structure part from *S. mobaraense* (45 amino acid residues) is deleted;

SEQ ID NO:33 = DNGAGE at the N-terminal of the pro-structure part from *S. mobaraense* (45 amino acid residues) is deleted;

SEQ ID NO:34 = RAP at the C-terminal of the pro-structure part from *S. mobaraense* (45 amino acid residues) is

modified to GPK;

SEQ ID NO:35 = RAP at the C-terminal of pro-structure part from *S. mobaraense* (45 amino acid residues) is modified to GPR;

SEQ ID NO:36 = GPSFRAP at the C-terminal of the pro-structure part from *S. mobaraense* (45 amino acid residues) is modified to GPK (FRAP is deleted and S at the C-terminal is modified into K);

SEQ ID NO:37 = GPSFRAP at the C-terminal of the pro-structure part from *S. mobaraense* (45 amino acid residues) is modified to GPR (FRAP is deleted and S at the C-terminal is modified into S);

SEQ ID NO:38 = the chimeric pro-structure part (56 amino acid residues) consisting of the partial pro-structure parts from *S. mobaraense* (15 amino acid residues) and the partial pro-structure parts *S. cinnamomeum* (41 amino acid residues).

[0035] Thus the pro-structure part may have replacement, deletion, insertion or addition of one or more amino acids as long as it has the recognition site specific to protease at given position.

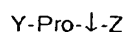
[0036] The N-terminal region of the protein generated by protease degradation may not be necessarily identical to that of the naturally occurring protein and one to several amino acids may be further added to or deleted from the naturally occurring protein.

[0037] Generally it is preferred that the resulting protein is cleaved at the appropriate same position as that of a naturally occurring protein in terms of its activity and it is more preferred that it is identical to the mature peptide of a naturally occurring protein. For example, the sequences of mature transglutaminases of *S. mobaraense* and *S. cinnamomeum* are shown in SEQ ID NO:5 and SEQ ID NO:43, respectively. Therefore the specific proteases which cleaves the propeptide at the position such that it generate the same protein as the naturally occurring mature protein are generally most preferable. However, for a particular objective the peptides having longer or shorter sequence of amino acid residue by from one to several residues at the N-terminal relative to that of a naturally occurring protein may possess more appropriate activity. Such proteases include, for example, Dispase (available from Boeringer Mannheim Co.) which can be commercially available and proteases obtained from the culture medium of microorganisms, such as, for example, the culture medium of actinomycetes. Such proteases may be used in a unpurified state or optionally may be used after purification to the appropriate purity.

[0038] An example of other suitable protease is SAMP45, a serine protease produced by *Streptomyces albogriseolus* (hereinafter it may be abbreviated as *S. albogriseolus*). In the case of the protransglutaminase from *S. mobaraense*, since SAMP45 predominantly cleaves between Ser at position 41 and Phe at position 42 of the pro-structure part as defined in SEQ ID NO: 3, the protein, which has the structure wherein the additional four amino acids of Phe-Arg-Ala-Pro of the C-terminal form the pro-structure part is added to the N-terminal of the naturally occurring mature transglutaminase shown in SEQ ID NO: 5, is generated. The present inventors confirmed that these proteins also had the activities of transglutaminase. The sequence of SAMP45 gene has been already determined and the amino acid sequence of the protein with the additional pro-structure part (proSAMP45) is shown in SEQ ID NO:39 (J. Bacteriol., 179, 430-438 (1997)), as well. When SAMP45 is allowed to act on the protransglutaminase in the form of the culture medium of *S. albogriseolus* or in the form of *S. Albogriseolus* cell, it can cleave the pro-structure part leaving a part thereof remained, resulting in the transglutaminase wherein almost all of the pro-structure part is removed. Alternatively the transglutaminase wherein a large part of the pro-structure part is removed can be similarly obtained by co-culturing a coryneform bacterium to which preproSAMP45 gene has been introduced with a coryneform bacterium that secretes protransglutaminase. Additionally the activation of the transglutaminase through the cleavage of the pro-structure part can be efficiently performed by similarly introducing the SAMP45 gene into the Coryneform bacterium into which the preprotransglutaminase gene has been introduced and by allowing to simultaneously secrete-produce the SAMP45 as well as the protransglutaminase.

[0039] Further mature transglutaminase identical to the naturally occurring transglutaminase can be obtained by using the proline-specific peptidase produced by *S. mobaraense* (svPEP), which has been found by the inventors, combined with SAMP45, which results in the removal of the four amino acids of Phe-Arg-Ala-Pro added at the N-terminal.

[0040] This svPEP is an enzyme that cleaves specifically the peptides or the peptide analogues represented by the following formula(I) at the site shown with ↓ in the formula, that is, at the carboxyl terminal side of the third or fourth proline residue from the N-terminal:



wherein Y represents an oligopeptide consisting of two or three amino acid residues and Z represents an amino acid, peptide, amide or ester.

[0041] More specifically this proline-specific peptidase is a proline-specific peptidase having the following properties

shown in (1)-(8):

(1) It cleaves at least one of the following proline containing peptides at the site shown with ↓, that is, at the carboxyl terminal side of the proline residue (wherein pNA is p-nitroanilide):

Ala-Ala-Pro- ↓ -pNA, Ala-Phe-Pro- ↓ -pNA, Phe-Arg-Ala-Pro- ↓ -pNA (identical to Phe-Arg-Ala Xaa (SEQ ID NO:68) (wherein Xaa represents Pro-pNA and pNA represents p-nitroanilide))

(2) It has the optimum pH of 6.0-6.5;

(3) It is stable at pH4-9;

(4) It has the optimum temperature of 25-30°C;

(5) It is stable below 20°C;

(6) Its activity is inhibited by phenylmethylsulfonyl fluoride, aminoethylbenzenesulfonyl fluoride hydrochloride;

(7) It has the isoelectric point of 10.2; and

(8) It has the molecular weight of approximately 50,000

**[0042]** For example, this svPEP can be prepared as described hereinafter. Actinomycetes which produces the peptidases having svPEP activities, for example, actinomycetes *S. mobaraense* IFO13819 is cultured according to the method conventionally used for the cultivation of actinomycetes. The culture medium for culturing actinomycetes IFO13819 may be the common medium containing conventional carbon sources, nitrogen sources, inorganic ions and others. Glucose, starch, sucrose and others can be used as the carbon sources. Peptone, yeast extract, meat extract, malt extract, ammonium salt and others are optionally used as the nitrogen sources if necessary. Cultures may be incubated under the aerobic condition which is appropriately controlled within, for example, the pH range of between pH 5.0 and 8.5 and the temperature range between 15 to 37°C. The culture period is usually as long as 1 to 10 days, although it depends on the temperature, pH and the type of medium. In principle, the culture may be terminated at the time when the maximum amount of the intended svPEP can be achieved.

**[0043]** After the incubation of the culture for the period described above, the purified svPEP preparation can be obtained by recovering the cells from the culture, washing them briefly, eluting the fraction containing svPEP from the surface of the cells and purifying the eluent with the combination of purification techniques as HPLC and FPLC well known to those skilled in the art which is conventionally used for purifying proteins such. The elution of svPEP fraction from the surface of the cells can be performed by shaking the cells in a buffer solution such as, for example, 0.1 M of sodium phosphate buffer (pH 7.0) for a given period as long as 1 to 5 hours. The temperature during the procedure is between 0 to about 5°C to prevent the inactivation of the enzyme. svPEP can be isolated and purified from the supernatant of the cultures, but it contains a lot of contaminated proteins and therefore it is more advantageous to elute and purify the protein from the surface of the washed cells.

**[0044]** The active fractions in each step can be confirmed by determining the activities of the enzyme in the fractions. The determination of the activities can be achieved using a combination of appropriate substrates and the detection method of the reaction products, for example, by reacting the enzymes with Ala-Ala-Pro-pNA, Ala-Phe-Pro-pNA, Phe-Arg-Ala-Pro-pNA as substrates and measuring the amount of the pNA (p-nitroanilide) released from the reaction to quantify the activities.

**[0045]** The genes encoding svPEP can be obtained by determining the partial amino acid sequence and designing appropriate probes, optionally after further purifying svPEP, which has been purified as previously described, by using reversed phase chromatography, etc. This procedure is well known to those skilled in the art. See, e.g., Molecular Cloning 2nd edition [J. Sambrook E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p9. 31 (1989)]. The sequence of the gene for svPEP obtained in this way and the entire sequence of the amino acid coded thereby are shown in SEQ ID NO: 41 and SEQ ID NO: 42, respectively, and the amino acid sequence of the mature protein of svPEP are shown in SEQ ID NO:40.

**[0046]** When svPEP is reacted on the protransglutaminase together with the protease in the form of medium of *S. mobaraense* or *S. mobaraense* cells, the pro-structure part can be cleaved completely, resulting in the mature transglutaminase of which pro-structure part is completely removed. Alternatively, the mature transglutaminase of which pro-structure part is completely removed can be similarly obtained by culturing a coryneform bacterium wherein pre-pro svPEP gene and a protease gene are introduced together with a coryneform bacterium which releases a protransglutaminase extracellularly by secretory production. Moreover a mature transglutaminase having the same structure as that of a naturally occurring form can be efficiently produced by introducing similarly both SAMP45 gene and svPEP gene into a coryneform bacterium to which pre-protransglutaminase gene has been introduced, and by allowing it to secrete-produce protransglutaminase and SAM45 as well as svPEP extracellularly or at the surface of the cells.

**[0047]** The method for introducing the genetic constructs that can be used in the present invention into a coryneform bacterium is not limited to particular methods and the methods generally used including, for example, the protoplast

method (Gene, 39, 281-286 (1985)), the electroporation method (Bio/Technology, 7, 1067-1070) (1989)), etc.. The resulting transformant can be cultured according to the conventional methods and conditions. For example, the transformant can be cultured with a conventional medium containing carbon sources, nitrogen sources and inorganic sources. Trace amount of organic nutrients such as vitamins and amino acids can be optionally added to the medium in order to achieve the growth to greater extent.

[0048] Carbohydrates such as glucose and sucrose as carbon sources, organic acids such as acetic acid, alcohols and others can be used. Gaseous ammonia, aqueous ammonia, ammonium salt and others can be used as nitrogen sources. As inorganic ions, calcium ion, magnesium ion, phosphorus ion, potassium ion, ferrous or ferric ion and others are optionally used as necessary. The culture is conducted for about 1 to 7 days under the aerobic condition in the appropriate range of pH between 5.0 and 8.5 and of the temperature between 15°C and 37°C. By culturing the transformant under such conditions, a large amount of an intended protein is produced intracellularly and is efficiently secreted extracellularly. Transglutaminase is generally known to be lethal when it is largely accumulated in the cells of microorganisms, but according to the present invention, transglutaminase is continuously produced without generating lethal effects, since the intracellularly produced transglutaminase is released extracellularly.

[0049] The proteins which are secreted in the medium according to the present invention can be isolated and purified from incubated culture medium according to the methods well known to those skilled in the art. For example, the proteins can be isolated and purified by removing the cells from the medium by centrifugation, etc., and then by using known appropriate methods such as salting-out, ethanol precipitation, ultrafiltration, gel filtration chromatography, ion-exchange column chromatography, affinity chromatography, medium high-pressure liquid chromatography, reversed-phase chromatography, hydrophobic chromatography or the combination thereof. The proteins secreted at the surface of the cells according to the present invention can be isolated and purified by using the methods well known to those skilled in the art, for example, by solubilizing them with increased salt concentrations or surfactants, and then using the similar methods to that for the proteins secreted in the medium. Additionally in some cases the proteins secreted at the surface of the cell may be used without solubilization, for example, as immobilized enzymes.

[0050] The present invention is further specifically described in the following Examples, which are not to be construed in any way as the limitation of the present invention.

#### Examples

##### Example 1 : Expression of prepro-transglutaminase derived from *S. mobaraense* IFO13819 in *C. glutamicum* ATCC13869

##### (1) Acquisition of the transglutaminase gene derived from *S. mobaraense* IFO13819

[0051] The sequence of transglutaminase gene derived from *S. mobaraense* DSMZ strain has been already determined [Eur. J. Biochem., 257, 570-576(1998)]. The primers shown in SEQ ID NO: 8 and SEQ ID NO: 9 were synthesized by reference to the sequence and the region encoding the sequence of mature transglutaminase was amplified using PCR method with the chromosomal DNA of *S. mobaraense* IFO13819 prepared according to the conventional procedure (the method of Saito and Miura [Biochim, Biophys. Acta, 72, 619(1963)]. For PCR reaction, Pyrobest DNA polymerase (Takarashuzo Co. Ltd.) was used and the reaction condition followed the protocol recommended by the manufacturer.

(SEQ ID NO: 8) 5'-GACTCCGACGACAGGGTCACCCCTCCCGCC-3'

(SEQ ID NO: 9) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

< sequence listing free text >

SEQ ID NO: 8 and SEQ ID NO: 9 : PCR primer

[0052] The DNA probe was then generated by reacting the amplified DNA fragment of about 1.0 kb with [ $\alpha$ -<sup>32</sup>P]dCTP using Random Primer DNA Labeling Kit Ver. 2 (Takarashuzo Co. Ltd.) according the protocol attached to the Kit. It was confirmed that the transglutaminase gene was present in the fragment of about 4 kb excised with restriction enzyme *Sac* I by Southern blot hybridization using the generated probe and the chromosomal DNA of *S. mobaraense* IFO13819 according to the conventional method, as described in Molecular Cloning 2nd edition [J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p9. 31 (1989)]. Accordingly, the fragment of about 4 kb which had

been generated by *SacI* digestion of the chromosomal DNA of *S. mobaraense* IFO13819 was recovered through agarose gel electrophoresis using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and was inserted into *Sac I* site of pUC18 (Takarashuzo Co. Ltd.) which was introduced into competent cells of *Escherichia coli* JM109 (Takarashuzo Co. Ltd.) to generate a library.

[0053] The bacterium strain was obtained which contains the plasmid where the transglutaminase gene fragment was cloned, by screening the library using the previously generated DNA probe for transglutaminase by colony hybridization as described in Molecular Cloning 2nd edition [J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p1. 90(1989)]. The plasmid was recovered from this strain and designated as pUITG. The sequence of the fragment cloned in pUITG was determined, which confirmed that the transglutaminase gene from *S. mobaraense* IFO13819 had the same sequence as that of the transglutaminase from *S. mobaraense* DSMZ strain.

[0054] The determination of the nucleotide sequence revealed that the *SacI* fragment of about 4kb was the incomplete DNA fragment from which the signal sequence (the pre-part) was partially deleted. Accordingly the cloning of the promoter region and the entire signal sequence region was attempted. The cloning was performed using TAKARA LA PCR in vitro Cloning kit (Takarashuzo Co. Ltd.) and the synthesized primers shown in SEQ ID NO: 10 and SEQ ID NO:11 according to the attached protocol.

(SEQ ID NO: 10) 5'-GTGACCCTGTCGTCGGAGTC-3'

(SEQ ID NO: 11) 5'-GGCATCCTGTCGAGCGGCTC-3'

<sequence listing free text>

SEQ ID NO: 10 and SEQ ID NO: 11: PCR primers for the promoter region and the signal sequence of *S. mobaraense*

[0055] Consequently when a cassette primer of *SacI* was used, the PCR-amplified fragment of about 800 bp was obtained and the sequencing of the fragment confirmed that the fragment contained the promoter region and the signal sequence region for the transglutaminase gene. Accordingly, the PCR-amplified fragment of about 800 bp was inserted into *SmaI* site of pVC7 described in JP-Kokai No. 9-070291 to obtain pVITGS5. Additionally plasmid pUITG was digested with *SacI*, the fragment of about 4kb was recovered through agarose electrophoresis, and the fragment was inserted to *SacI* site of pVITGS5 to construct plasmid pVITGC which comprises the full-length transglutaminase gene. The determination of the nucleotide sequence was performed using Dye Terminator Cycle Sequencing kit (PE Applied Biosystems) and DNA Sequencer 373A (PE Applied Biosystems). The sequence of the preprotransglutaminase gene is shown in SEQ ID NO: 12, wherein the N-terminal 31 amino acids sequence was believed to be the signal sequence (the pre-part). The amino acid sequence of the preprotransglutaminase is shown in SEQ ID NO: 13.

(2) Conversion of the promoter region of transglutaminase gene

[0056] The sequence of the gene for PS2 which is a surfaced protein of *C. glutamicum*, has been already determined [Mol. Microbiol., 9, 97-109(1993)]. Primers shown in SEQ ID NO: 14 and SEQ ID NO:15 were synthesized on referring to the sequence, and the region which comprises the promoter at the 5'-upstream region of the initiation codon of PS2 protein gene was amplified using PCR method from the chromosomal DNA of *C. glutamicum* ATCC13869 prepared according to a conventional method.

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 15)

5'-GAGCTCTCCGGCGTATGCGCATAGAGGCGAAGGCTCCTTGAATA-3'

<sequence listing free text>

SEQ ID NO: 14 and SEQ ID NO:15; PCR primers

[0057] On the other hand, primers shown in SEQ ID NO: 16 and SEQ ID NO: 9 were synthesized based on the sequence of the transglutaminase gene determined in Example 1(1), and the region of the preprotransglutaminase gene was amplified using PCR method from pUITG obtained in Example1(1).

(SEQ ID NO: 16) 5'-ATGCGCATACGCCGGAGAGCTCTCGTCTTC-3'

<sequence list free text>

SEQ ID NO: 16: PCR primer

[0058] Then, the fusion gene of transglutaminase fused with the additional pre-pro structure part, which was ligated to the region comprising the promoter of the cell surface protein gene from *C. glutamicum* ATCC13869, was amplified by performing cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using the mixture of 1 µl of each of the PCR solution of the amplified region comprising the promoter of PS2 gene of *C. glutamicum* ATCC13869 and the amplified pre-protransglutaminase gene region, as the templates. The amplified fragment of about 1.8 kb was detected by agarose gel electrophoresis. This fragment was recovered from the agarose gel with EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of pVC7 as described in JP-Kokai No. 9-070291 to obtain pVKPTG0. The nucleotide sequence of the inserted fragment was determined according to the method described above and it was confirmed that the fusion gene was constructed as expected.

(3) Expression of the pre-protransglutaminase gene in *C. glutamicum* ATCC13869

[0059] *C. glutamicum* ATCC13869 was transformed with the pVITGC constructed in Example 1(1) (both the promoter and the pre-protransglutaminase gene were derived from *S. mobaraense*) or with the pVKPTGO constructed in Example 1(2) (the promoter was derived from PS2 gene of *C. glutamicum* ATCC13869 and the pre-protransglutaminase gene was derived from *S. mobaraense*) and the strains grown on the CM2S agar medium comprising 5 mg/l of chloramphenicol (10 g of yeast extract, 10g of tryptone, 5 g of sucrose, 5 g of NaCl, 5 g of agar per liter of distilled water) were selected. The selected *C. glutamicum* ATCC13869 harboring pVITGC or pVKPTGO was cultured in MM culture medium (30 g of glucose, 0.4 g of magnesium sulfate heptahydrate, 30 g of ammonium sulfate, 1 g of potassium dihydrogenphosphate, 0.01 g of ferrous sulfate heptahydrate, 0.01 g of manganese(II) sulfate pentahydrate, 200 µg of thiamine hydrochloride, 500 µg of biotin, 0.15 g of DL-methionine, 50 g of calcium carbonate per liter of distilled water, adjusted to pH 7.5) comprising 5 mg/l of chloramphenicol at 30°C for 48 hours, respectively. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then to Western blot with anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994) according to the conventional method (for example, the general procedure as described in J. Sambrook et al. (1989)(supra)).

[0060] Consequently, the secretion of transglutaminase could not be detected. From the above results, it was confirmed that the signal sequence of transglutaminase from *S. mobaraense* did not function in *C. glutamicum* ATCC13869.

Example 2: Secretory production of mature transglutaminase using the fusion gene encoding the signal peptide of the cell surface protein of *Corynebacterium glutamicum* (*C. glutamicum* ATCC13869) and the mature transglutaminase derived from *S. mobaraense* IFO13819

(1) Construction of the transglutaminase gene containing the signal sequence of cell surface protein of *C. glutamicum* ATCC13869

[0061] The sequence of the gene of PS2 which is the cell surface protein of *C. glutamicum* has been already determined [Mol. Microbiol., 9, 97-109(1993)]. Primers shown in SEQ ID NO: 14 and SEQ ID NO:17 were synthesized on referring to the sequence, and the region encoding the N-terminal 44 amino acid residues (30 amino acid residues of the signal peptide and 14 amino acid residues of the mature cell surface protein) of the protein corresponding to PS2 and 5'-upstream region containing the promoter region were amplified using PCR method with the chromosomal DNA of *C. glutamicum* ATCC13869 prepared according to the method described in Example1(2). The primer shown in SEQ ID NO: 17 also comprises the sequence encoding the amino acid sequence from the N-terminal region of the mature



transglutaminase in order to construct the fusion gene fused with transglutaminase.

(SEQ ID NO: 14) 5'- AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO:17)

5'-GGGGTGACCCTGTCGTCGGAGTCGTTGAAGCCGTTGTTGATGTTGAA-3'

<sequence listing free text>

SEQ ID NO: 17: PCR primer

[0062] On the other hand, primers shown in SEQ ID NO: 8 and SEQ ID NO: 9 were synthesized based on the sequence of the transglutaminase gene determined in Example1(1) and the region of mature transglutaminase gene was amplified using PCR method with pUITG obtained in Example1(1).

[0063] The fusion gene of the mature transglutaminase, which was ligated to the region encoding the N-terminal 44 amino acid residues of *C. glutamicum* ATCC13869 and to the 5'-upstream region comprising the promoter gene of the cell surface protein gene, was amplified by performing cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using the mixture of 1 µl of PCR solution of the amplified region encoding the N-terminal 44 amino acid residues of the protein corresponding to PS2 of *C. glutamicum* and the 5'-upstream region containing the promoter, and 1 µl of PCR solution of the amplified mature transglutaminase gene region, as the templates.

[0064] The amplified fragment of about 1.7 kb was detected by agarose electrophoresis. This fragment was recovered from the agarose gel using EASYTRAP Ver. 2 (Takara Shuzo Co. Ltd.) and inserted into SmaI site of the pVC7 described in JP-Kokai No. 9-070291 to obtain pVKPTG3. The nucleotide sequence of the inserted fragment was determined according to the method described above and it was confirmed that the expected fusion gene was constructed.

[0065] Additionally, the fusion mature transglutaminase gene of about 1.7 kb, which had been ligated to the region encoding the N-terminal 44 amino acid residues from *C. glutamicum* ATCC13869 and the 5'-upstream region comprising the promoter of the cell surface protein gene, was excised by digesting pVKPTG3 with KpnI and XbaI and recovered using agarose electrophoresis. This fragment was inserted into the KpnI-XbaI site of pPK4 described in JP-Kokai No. 9-322774 to construct pPKTG3.

(2) Secretion of mature transglutaminase using the signal sequence of the cell surface protein of *C. glutamicum* ATCC13869

[0066] *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVKTG3 or pPKTG3 (in both cases the gene comprising the promoter and the gene encoding signal peptide and the N-terminal 14 amino acid residues were derived from *C. glutamicum* ATCC13869, and the mature transglutaminase gene was derived from *S. morabaraense*) and the strains grown on the CM2S agar medium comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin were selected. The selected *C. glutamicum* ATCC13869 containing pVITG3 or pVKPTG3 was then cultured in liquid MM culture medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin at 30°C for 48 hours, respectively. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot was performed according to a conventional method with anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994). As a result, a small amount of secreted transglutaminase having the similar molecular weight to that of the mature transglutaminase could be detected in the supernatant of the culture of both strains.

Example 3: Secretory production of pro-transglutaminase using pro-transglutaminase fusion gene (heterologously fused prepro-transglutaminase fusion gene) derived from *S. mobaraense* IFO13819 ligated to the signal peptide of cell surface protein of *C. glutamicum* ATCC13869

(1) Construction of transglutaminase gene (heterologously fused prepro-transglutaminase fusion gene) containing the additional pro-structure part with the signal peptide of cell surface protein of *C. glutamicum* ATCC13869

[0067] Primers shown in SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20 and SEQ ID NO: 21 were synthesized on referring to the sequence of the gene of PS2 which was the cell surface protein of *C. glutamicum* [Mol. Microbiol., 9, 97-109(1993)]. The coding region for the N-terminal 30, 31, 44 or 68 amino acid residues (the region comprising 30 amino acid residues of the signal peptide) and the 5'-upstream region containing the promoter region of the protein corresponding to PS2 were amplified by PCR method using the combination of SEQ ID NO: 14 and SEQ ID NO: 18, or of SEQ ID NO: 14 and SEQ ID NO: 19, or of SEQ ID NO: 14 and SEQ ID NO: 20, or of SEQ ID NO: 14 and SEQ ID NO: 21 from the chromosomal DNA of *C. glutamicum* ATCC13869 prepared according to the method described in Example 1(2).

[0068] Primers shown in SEQ ID NO: 18, SEQ ID NO: 19 and SEQ ID NO: 21 comprise the sequences encoding the N-terminal amino acids of pro-transglutaminase in order to construct the fusion gene fused with the transglutaminase having the pro-structure part.

(SEQ ID NO: 18)

5'-CTTCGTCTCTTCCCCCGCGCCATTGTCAGCGAATGCTGGGATAGCAACGCC-3'

(SEQ ID NO: 19)

5'-

CTTCGTCTCTTCCCCCGCGCCATTGTCCTGAGCGAATGCTGGGATAGCTAC-3'

(SEQ ID NO: 20)

5'-CTTCGTCTCTTCCCCCGCGCCATTGTCGTTGAAGCCGTTGTTGATGTTGAA-

3'

(SEQ ID NO: 21)

5'-

CTTCGTCTCTTCCCCCGCGCCATTGTCAGTCAGGTCGCGGAGGGTTTCCTC-

3'

<sequence listing free text>

SEQ ID NO: 18 to SEQ ID NO: 21: PCR primers

[0069] On the other hand, primers shown in SEQ ID NO: 22 and SEQ ID NO: 9 were synthesized based on the sequence of the transglutaminase gene determined in Example 1(1) and the pro-transglutaminase gene region was

amplified using PCR method with pUITG obtained in Example 1(1).

(SEQ ID NO: 22) 5'-GACAATGGCGCGGGGGAAGAGACGAAGTCC-3'

<sequence listing free text>

SEQ ID NO: 22: PCR primer

[0070] Then the heterologously fused pro-transglutaminase gene ligated to the respective region encoding its N-terminal 30, 31, 44 and 68 amino acid residues and the 5'-upstream region comprising the promoter region of the protein gene corresponding to PS2 from *C. glutamicum* ATCC13869, that is, the fragments of heterologously fused prepro-transglutaminase genes which were ligated to the promoter of the gene for cell surface protein of *C. glutamicum* ATCC13869, was amplified by performing cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using the mixture which comprises 1 µl of PCR solution of the 5'-upstream region containing the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum* ATCC13869 and each of the amplified region encoding N-terminal 30, 31, 44 or 68 amino acid residues of the protein, and 1 µl of PCR solution of the amplified region of the gene for the transglutaminase having the pro-structure part, as the templates.

[0071] The amplified fragments ranging about 1.8 kb to 1.9 kb was detected by agarose electrophoresis. These fragments were recovered from the agarose gels with EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of pVC7 as described in JP-Kokai No. 9-070291 to obtain pVKPTG1, pVKPTG2, pVKPTG3 and pVKPTG4, respectively. The nucleotide sequences of the inserted fragments were determined according to the forementioned method and it was confirmed that the expected fusion genes were expected.

[0072] Additionally, the fusion genes of about 1.8 kb to 1.9 kb of transglutaminase having the pro-structure parts, which was ligated to the respective regions encoding the 30, 31, 44 and 68 amino acid residues and the 5'-upstream region comprising the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum*, was excised by digesting pVKPTG1, pVKPTG2, pVKPTG3 and pVKPTG4 with KpnI and XbaI and were recovered by agarose electrophoresis. These fragments were inserted into KpnI-XbaI site of pPK4 described in JP-Kokai No. 9-322774 to construct pPKPTG1, pPKPTG2, pPKPTG3 and pPKPTG4.

(2) Secretion of pro-transglutaminase with the signal sequence of the cell surface protein of *C. glutamicum* ATCC13869

[0073] *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVKPTG1, pVKPTG2, pVKPTG3, pVKPTG4, pPKPTG1, pPKPTG2, pPKPTG3 or pPKPTG4 and the strains grown on the CM2S agar medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pVKPTG1, pVKPTG2, pVKPTG3, pVKPTG4, pPKPTG1, pPKPTG2, pPKPTG3 or pPKPTG4 was then cultured in MM culture medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin at 30°C for 48 hours, respectively. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot was performed using anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994) according to the conventional method. As a result, the secretion of the similar amount of transglutaminase having the pro-structure part was confirmed for both of the vectors, pVC7 or pPK4, and the significant difference in the secreted amount was observed depending on the length of N-terminal amino acid residues of the mature form of the protein corresponding to PS2. The representative secreted amounts are shown in Table 1.

Table 1.

The secreted amount of pro-transglutaminase using the signal sequence of the cell surface protein of <i>C. glutamicum</i> ATCC13869	
plasmid	pro-transglutaminase (mg/l)
pPKPTG1	78
pPKPTG4	210

(3) Cleavage of the pro-transglutaminases by Dispace digestion and the detection of their activities

[0074] To the supernatant of the culture of *C. glutamicum* ATCC13869 harboring pVKPTG1, pVKPTG2, pVKPTG3, pVKPTG4, pPKPTG1, pPKPTG2, pPKPTG3 or pPKPTG4, the protease, Dispace (Boeringer Mannheim Co. Ltd.) was

added at a ratio of substrate : enzyme = 1:1 and the reaction was maintained at 37°C, pH 7.5 for 1 hour. After the Dispa digestion, the cleavage of the pro-transglutaminases was confirmed by SDS-PAGE, and the similar specific activities (about 20 U/mg) to that of the naturally occurring transglutaminase was confirmed, as determined by hydroxamate method [J. Biol. Chem., 241, 5518-5525(1966)].

Example 4: Secretory production of pro-transglutaminase using the fusion gene having the sequence encoding the signal sequence of the cell surface protein of C. ammoniagenes and the pro-transglutaminase derived from S. mobaraense IFO13819

(1) Construction of the transglutaminase gene having the additional pro-structure part and the signal sequence of the cell surface protein of C. ammoniagenes (heterologously fused preprotransglutaminase fusion gene)

[0075] Primers shown in SEQ ID NO: 23 and SEQ ID NO: 24 were synthesized on referring to the sequence of the gene of the cell surface protein (SlpA) [JP-Kokai No. 10-108675] of C. ammoniagenes and the region comprising the 5'-upstream region containing the promoter region of the cell surface protein (SlpA) gene and the region encoding its N-terminal 25 amino acid residues (the signal peptide) were amplified using PCR method from the chromosomal DNA of C. ammoniagenes prepared according to the conventional method. The primer shown in SEQ ID NO: 24 also comprises the sequence encoding the N-terminal amino acids of the pro-transglutaminase in order to construct the fusion gene fused with the pro-transglutaminase.

(SEQ ID NO: 23) 5'-GCCCAGAAGCCCAAATTGAGATTT-3'

(SEQ ID NO: 24)

5'-

CTTCGTCTCTTCCCCGCGCCATTGTCTGCCGTTGCCACAGGTGCGGCCAGC

3'

<sequence listing free text>

SEQ ID NO: 23 and SEQ ID NO: 24: PCR primers

[0076] The fusion transglutaminasetransglutaminase gene containing the additional pro-structure part which was ligated to the region encoding the N-terminal 25 amino acid residues of C. ammoniagenes and the 5'-upstream region comprising the promoter region of the cell surface protein (SlpA) gene (heterologously fused prepro-transglutaminase gene) was amplified by performing cross-over PCR with SEQ ID NO: 23 and SEQ ID NO: 9 using the mixture as the templates containing 1 µl of PCR solution of the amplified 5'-upstream region containing the promoter region of the gene of the cell surface protein (SlpA) and the amplified region encoding the N-terminal 25 amino acid residues of the cell surface protein (SlpA) of C. ammoniagenes, and 1 µl of PCR solution of the region of the gene for the transglutaminase having the additional pro-structure part which had been amplified in Example 3(1). The amplified fragment of about 1.7 kb was detected by agarose electrophoresis. This fragment was recovered from agarose gel using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and was inserted into SmaI site of pVC7 to obtain pVSPTG1.

(2) Conversion of the promoter region: Ligation with the promoter of the cell surface protein gene of C. glutamicum, ATCC13869

[0077] Primers shown in SEQ ID NO: 14 and SEQ ID NO: 25 were synthesized on referring to the sequence of the gene of PS2 which is the cell surface protein [Mol. Microbiol.; 9, 97-109(1993)] of C. glutamicum. The 5'-upstream region comprising the promoter region of the gene for the protein corresponding to PS2 was amplified using PCR method from the chromosomal DNA of C. glutamicum ATCC13869 prepared according to the method in Example 1(2). The primer shown in SEQ ID NO: 25 also comprises the sequence encoding the N-terminal amino acids of the signal sequence of the cell surface protein (SlpA) of C. ammoniagenes in order to construct the fusion gene fused of the

transglutaminase gene having the pro-structure part fused with the signal sequence of the superficial zone protein (SlpA) of *C. ammoniagenes* (heterologously fused prepro-transglutaminase fusion gene).

(SEQ ID NO: 25)

5'-

CGCAGCCAGCGATTTTCATGCGTTTCATAGAGGCGAAGGCTCCTTGAATAGGT-

3'

<sequence listing free text>

SEQ ID NO: 25; PCR primer

[0078] On the other hand, primers shown in SEQ ID NO: 26 and SEQ ID NO: 9 were synthesized based on the sequence of the transglutaminase fusion gene having the additional pro-structure part, which contained the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes*, and the region of the transglutaminase having the additional pro-structure part, which contained the signal sequence of cell surface protein (SlpA) of *C. ammoniagenes*, was amplified using PCR method from pVSPTG1 obtained in Example 4(1).

(SEQ ID NO: 26) 5'-ATGAAACGCGATGAAATCGCTGGCTGCGGCG-3'

<sequence listing free text>

SEQ ID NO: 26; PCR primer

[0079] The fusion gene of transglutaminase having the pro-structure part, which was ligated to the region encoding the N-terminal 25 amino acid residues of the cell surface protein (SlpA) of *C. ammoniagenes* and to the 5'-upstream region containing the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum* ATCC13869, was then amplified by performing cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using the mixture comprising 1 µl of PCR solution of the amplified 5'-upstream region containing the promoter region of the gene for the protein corresponding to PS2 of *C. glutamicum* and 1 µl of PCR solution of the amplified region of the gene for the transglutaminase having the pro-structure part which had the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* (heterologously fused prepro-transglutaminase gene).

[0080] The amplified fragment of about 1.8 kb was detected by agarose electrophoresis. This fragment was recovered from the agarose gel using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of pVC7 described in JP-Kokai No. 9-070291 to obtain pVKSTG1. The nucleotide sequence of the inserted fragment was determined according to the forementioned method and it was confirmed that the expected fusion gene was constructed.

[0081] The fusion gene of about 1.8 kb of fusion gene for transglutaminase having the pro-structure, which was ligated to the region encoding the N-terminal 25 amino acid residues (signal peptide) of the cell surface protein (SlpA) of *C. ammoniagenes* and comprised the 5'-upstream region containing the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum* ATCC13869, was excised by digesting pVKSTG1 with KpnI and XbaI and was recovered using agarose electrophoresis. This fragment was inserted into KpnI-XbaI site of pPK4 described in JP-Kokai No. 9-322774 to construct pPKSTG1. Both plasmids, pVKSTG1 and pPKSTG1 comprised the promoter from PS2 gene of *C. glutamicum* ATCC13869, the signal peptide gene from SlpA of *C. ammoniagenes* and the transglutaminase gene from *S. mobaraense*.

(3) Conversion into *E. coli* tac promoter

[0082] Primers shown in SEQ ID NO: 27 and SEQ ID NO: 28 were synthesized based on the sequence of plasmid pKK223-3 (Amersham Pharmacia Co. Ltd.) wherein *E. coli* tac promoter was cloned. The region corresponding to tac promoter was amplified using PCR method from pKK223-3 DNA. The primer shown in SEQ ID NO: 28 also comprises the sequence encoding the N-terminal amino acid sequence of the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* in order to construct the fusion gene having the pro-structure part, which contained the signal

sequence of the cell surface protein (SlpA) of *C. ammoniagenes* (heterologously fused prepro-transglutaminase gene).

(SEQ ID NO: 27) 5'-GGATCCGGAGCTTATCGACTGCACG-3'

(SEQ ID NO: 28)

5'-

CGCAGCCAGCGATTTTCATGCGTTTCATAATTCTGTTTCCTGTGTGAAATTGT-3'

<sequence listing free text>

SEQ ID NO: 27 and SEQ ID NO: 28: PCR primers

**[0083]** The fusion gene for transglutaminase having the additional pro-structure part, which was ligated to the region encoding the N-terminal 25 amino acid residues of the superficial zone protein(SlpA) of *C. ammoniagenes* and which contained tac-promoter (heterologously fused prepro-transglutaminase gene), was amplified by performing cross-over PCR with SEQ ID NO: 27 and SEQ ID NO: 9 using the mixture of 1 µl of PCR solution of the amplified region corresponding to tac-promoter and 1 µl of PCR solution of the amplified region of the gene for transglutaminase having the pro-structure part, which contained the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes*, as the templates. The amplified fragment of about 1.5 kb was detected by agarose electrophoresis. This fragment was recovered from the agarose gel by EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of the pVC7 as described in JP-Kokai No. 9-070291 to obtain pVTSPTG1. The nucleotide sequence of the inserted fragment was determined according to the forementioned method and it was confirmed that the expected fusion gene was constructed.

**[0084]** The fusion gene about 1.5 kb for transglutaminase having the pro-structure part, which was ligated to the region encoding the N-terminal 25 amino acid residues of the cell surface protein(SlpA) of *C. ammoniagenes* and tac promoter, was excised by digesting pVTSPTG1 with KpnI and XbaI and was recovered using agarose electrophoresis. This fragment was inserted into KpnI-XbaI site of pPK4 described in JP-Kokai No. 9-322774 to construct pPTSPTG1. Both plasmids pVTSPTG1 and pPTSPTG1 comprised tac-promoter derived from *E. coli*, the signal peptide gene derived from SlpA of *C. ammoniagenes* and the pro-transglutaminase gene derived from *S. mobaraense*.

(4) Secretion of the pro-transglutaminase using the signal sequence of cell surface protein of *C. ammoniagenes*

**[0085]** *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVKSPTG1, pVTSPTG1, pPKSPTG1, or pPTSPTG1 and the strains grown on the CM2S agar medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pVKSPTG1, pVTSPTG1, pPKSPTG1, or pPTSPTG1 was then cultured in the MM culture medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin at 30°C for 48 hours, respectively. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot was performed with anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994) according to the conventional method. As a result, the similar amount of transglutaminase was confirmed to be secreted for either of the vectors, pVC7 or pPK4. The representative amounts of the secretion are shown in Table 2.

Table 2.

The secreted amount of pro-transglutaminase using the signal sequence of cell surface protein of <i>C. ammoniagenes</i> ATCC13869	
plasmid	protransglutaminase (mg/l)
pPKSPTG1	102
pPTSPTG1	74

## (5) Cleavage of protransglutaminase by Dispase digestion and the detection of the activity

[0086] To the supernatant of the culture of *C. glutamicum* ATCC13869 harboring pVKSPTG1, pVTSPTG1, pPKSPTG1, or pPTSPTG1, a protease Dispase (Boehringer Mannheim Co. Ltd.) was added at a ratio of substrate : enzyme = 1:1 and the reaction was maintained at 37°C pH 7.5 for 1 hour. After Dispase digestion, the reaction mixture was subjected to SDS-PAGE to confirm the cleavage of pro-transglutaminase having the pro-structure part, and the similar specific activity (about 20 U/mg) to that of the naturally occurring transglutaminase was confirmed to be contained, after determining the transglutaminase activity by hydroxamate method.

Example 5: Cleavage of protransglutaminase using the culture medium or the cells of *S. mobaraense* and the detection of the activity

## (1) Cleavage of protransglutaminase using the culture medium and the detection of the activity

[0087] *S. mobaraense* IFO13819 strain was cultured in ISP2 culture medium (4 g of yeast extract, 10 g of malt extract, 4 g of glucose per liter of distilled water, adjusted to pH7.3) at 30 °C for 24 hours. To 10 ml of the culture medium, the supernatant of the culture of *C. glutamicum* ATCC13869 harboring pVKSPTG1, pVTSPTG1, pPKSPTG1, or pPTSPTG1, which was also used in Example 4(5) and where pro-transglutaminase was accumulated, was added after filtration by a membrane filter and the culture medium was maintained at 30°C for 6 hours. It was then subjected to SDS-PAGE to confirm the cleavage of transglutaminase having the pro-structure part. The activity of the transglutaminase was confirmed, which had the similar specific activity (about 20 U/mg) to that of the naturally occurring transglutaminase as determined by hydroxamate method. Further it was semi-dry blotted onto polyvinylidene-difluoride (PVDF) membrane after SDS-PAGE (Structural analysis of proteins for gene cloning, Tokyo Kagaku Dojin(1993)). After blotting, the PVDF membrane was stained with Coomassie Brilliant Blue, de-stained and air-dried. The portion containing the mature transglutaminase was excised and analyzed for the N-terminal amino acid sequence using Protein Sequencer (Model 476A, Parkin Elmer Co. Ltd.). As a result, it was confirmed that the protein had the same amino acid sequence to that of the naturally occurring mature transglutaminase shown in SEQ ID NO: 5.

(2) Cleavage of transglutaminase with the pro-structure part using the cells of *S. mobaraense* IFO13819 strain

[0088] *S. mobaraense* IFO13818 strain was cultured in ISP2 culture medium at 30°C for 24 hours. The cells were harvested by centrifugation of 10 ml of the culture medium and were washed twice with saline. The cells finally harvested were suspended in 10 ml of saline. To the suspensions, 10 ml of the supernatant of the culture of *C. glutamicum* ATCC13869 harboring pVKSPTG1, pVTSPTG1, pPKSPTG1, or pPTSPTG1 which was also used in Example 4(5) and where the pro-transglutaminase was accumulated, was added after filtration, and the mixture was maintained at 30°C for 6 hours. Then it was subjected to SDS-PAGE and the cleavage of transglutaminase having the pro-structure part was confirmed, and the transglutaminase activity showing the similar specific activity (about 20 U/mg) to that of the naturally occurring transglutaminase was confirmed to be contained as determined by hydroxamate method. Further it was semi-dry blotted onto polyvinylidene-difluoride (PVDF) membrane after SDS-PAGE process (Structural analysis of proteins for gene cloning, Tokyo Kagaku Dojin(1993)). After the blotting, the PVDF membrane was stained with Coomassie Brilliant Blue, de-stained and air-dried. The portion containing mature transglutaminase was excised and were analyzed for the N-terminal amino acid sequence using a protein sequencer. As a result, it was confirmed that the protein had the same amino acid sequence to that of the naturally occurring mature transglutaminase shown in SEQ ID NO: 5.

Example 6: Secretory production of pro-transglutaminase using the fusion gene containing the sequence encoding the signal sequence of the cell surface protein of *C. ammoniagenes* and the protransglutaminase derived from *Streptovercillium cinnamomeum* IFO12852

(1) Construction of the fusion gene comprising the sequence encoding the signal sequence of the cell surface protein of *C. ammoniagenes* and the sequence encoding the pro-transglutaminase derived from *S. cinnamomeum* IFO12852

[0089] The sequence of the transglutaminase gene of *S. cinnamomeum* IFO12852 has been determined [Japanese Patent Application No.11-295649]. The region from position 1 to position 32 in the amino acid sequence is presumed to be the sequence for the pre-part, from position 33 to position 86 is presumed to be the sequence for the pro-part and from position 87 to position 416 is presumed to be the sequence for the mature transglutaminase sequence. Putative sequences of the pro-structure part and the mature protein are shown in SEQ ID NO: 4 and SEQ ID NO: 43, respectively. Additionally *Escherichia coli* AJ13669 which had been transformed with the plasmid pUJ-MTG containing



the gene has been deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology on Oct. 14, 1999 as FERM P-17602 and has been transferred to the deposit under the Budapest Treaty on August 28, 2000, and the deposit number of FERM BP-7287 has been allotted.

[0090] The region of 3.5 kb covering the full-length of the prepro-transglutaminase gene was firstly excised from pUJ-MTG with restriction enzyme BamHI, and pUCSCTG was generated wherein the region was inserted into BamHI site of pUC19.

[0091] Primers shown in SEQ ID NO: 44 and SEQ ID NO: 45 were synthesized, and the region of the gene comprising the pro-transglutaminase derived from *S. cinnamomeum* IFO12852 was amplified by PCR method using pUCSCTG as the template as previously described.

(SEQ ID NO: 44) 5'-GGC GAT GGG GAA GAG AAG GGG-3'

(SEQ ID NO: 45) 5'-GGC GGA TCC TVG CGT CGA GAG GCG TGG ACT GA-3'

<sequence listing free text >

SEQ ID NO: 44 and SEQ ID NO: 45: PCR primers

[0092] The region, which comprises the 5'-upstream region containing the promoter region of PS2 gene which is the cell surface protein of *C. glutamicum* and the region containing the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*, was then amplified by performing PCR using the combination of SEQ ID NO: 46 and SEQ ID NO: 47 from pPKSPTG1 as the template which was constructed in Example 4(2).

[0093] The primer shown in SEQ ID NO: 47 also comprises the sequence encoding the N-terminal amino acid sequence of pro-transglutaminase derived from *Streptovorticillium cinnamomeum* IFO12852 in order to construct the fusion gene with the transglutaminase derived from *Streptovorticillium cinnamomeum* IFO12852.

(SEQ ID NO: 46) 5'-TAC GAA TTC GAG CTC GGT ACC-3'

(SEQ ID NO: 47) 5'-CCC CTT CTC TTC CCC ATC GCC TGC CGT TGC CAC AGG  
TGC GGC C -3'

<sequence listing free text>

SEQ ID NO: 46 and SEQ ID NO: 47: PCR primers

[0094] The fragment of the heterologously fused prepro-transglutaminase gene, which was ligated to the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and the 5'-upstream region comprising the promoter region of PS2 gene, was amplified by performing cross-over PCR with SEQ ID NO: 46 and SEQ ID NO: 45 using the mixture comprising 1 µl of PCR solution of the amplified region encoding the gene for the pro-transglutaminase derived from *C. cinnamomeum* IFO12852 and 1 µl of PCR solution of the amplified 5'-upstream region containing the promoter region of the PS2 gene and the amplified region comprising the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*, as the templates.

[0095] The amplified fragment of about 1.8 kb was detected by agarose electrophoresis.

[0096] This fragment was digested with EcoRI and BamHI, and then recovered from the agarose gel and inserted into EcoRI-BamHI site of the pUC19 to obtain pUKSPTG2'. The sequence of the inserted fragment was determined according to the forementioned method and it was confirmed that the fusion gene was constructed as expected. This pUKSPTG2' was digested with EcoRI and bluntended with Blunting Kit (Takarashuzo Co. Ltd.), and XbaI linker (Takarashuzo Co. Ltd.) having the sequence 5'-CTCTAGAG-3' wherein 5'-terminal was phosphorylated was then inserted and re-cyclized to construct pUKSPTG2. The fused preprotransglutaminase gene of about 1.8 kb (the protransglutaminase gene was derived from *S. cinnamomeum* IFO12852) was excised by digesting pUKSPTG2 with XbaI and was

recovered using agarose electrophoresis. These fragments were inserted into XbaI site of pPK4 described previously to construct pPKSPTG2.

[0097] The preprotransglutaminase having a chimeric pro-structure part, wherein the N-terminal of the pro-structure part was partially replaced by the pro-structure part of *S. mobaraense*, was constructed (the mature transglutaminase gene and the part of the pro-structure part were derived from *S. cinnamomeum* IFO12852).

[0098] Firstly, the fragment of about 1.8 kb containing the prepro-transglutaminase gene of EcoRI-BamHI was excised from the plasmid pPKSPTG1 (for the expression of the pro-transglutaminase derived from *S. mobaraense* IFO13819) which was constructed in Example 4(2), and the fragment was inserted into EcoRI-BamHI site of pUC19 (pUKSPTG1). The fragment of about 1.2 kb was excised by digesting pUKSPTG1 with AatII, and pUKSPTG2' was also digested with AatII to prepare the fragment of about 3.3 kb removing the fragment of about 1.2 kb. This fragment of about 3.3 kb was ligated to the AatII fragment of about 1.2 kb derived from pUKSPTG1, and clones wherein the AatII fragment was inserted were selected according to the conventional genetic engineering techniques. In order to determine which orientation the AatII fragment was inserted into the clones, they were serially sequenced and the clones wherein the fragment was inserted in the desired orientation were selected (pUKSPTG3'). Moreover the EcoRI site of pUKSPTG3' was also blunt-ended as with pUKSPTG2' and XbaI linker was inserted to construct pUKSPTG3. Further the 1.8kb XbaI fragment excised from pUKSPTG3 was inserted into XbaI site of pPK4 to construct pPKSPTG3.

(2) Secretion of the pro-transglutaminase derived from *Streptovercillium cinnamomeum* IFO12852 using the signal sequence of the cell surface protein from *C. ammoniagenes*

[0099] *C. glutamicum* ATCC13869 was transformed using the plasmid pPKSPTG2 or pPKSPTG3, and the strains which grew on the CM2S agar medium described above comprising 25 mg/l of kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pPKSPTG2 or pPKSPTG3 was then cultured respectively in MMTG liquid culture medium (60 g of glucose, 0.4 g of magnesium sulfate heptahydrate, 30 g of ammonium sulfate, 1 g of potassium dihydrogenphosphate, 0.01 g of ferrous sulfate heptahydrate, 0.01 g of manganese(II) sulfate pentahydrate, 450 µg of thiamine hydrochloride, 450 µg of biotin, 0.15 g of DL-methionine, 50 g of calcium carbonate per liter of distilled water, adjusted to pH 7.5) containing 25 mg/l of kanamycin at 30°C for 3 days. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot analysis was performed according to the conventional method with anti-transglutaminase antibody as described previously. The antibody is an antibody for the transglutaminase derived from *S. mobaraense*, but it also showed the reactivity to the transglutaminase derived from *S. cinnamomeum*. Consequently the secretion of the transglutaminase having the pro-structure part derived from *S. cinnamomeum* IFO12852 was confirmed (about 30 to 50 mg/l).

Example 7: Secretory production of transglutaminase by replacing the pro-structure part of the pro-transglutaminase derived from *S. mobaraense* IFO13819 by the pro-structure part derived from *S. cinnamomeum* IFO12852 (generation of the hybrid)

[0100] Primers shown in SEQ ID NO: 14 and SEQ ID NO: 48 were synthesized and the 5'-upstream region comprising the promoter region of PS2 gene of *C. glutamicum* ATCC13869 and the region coding the signal sequence of the cell surface protein of *C. ammoniagenes* and also the pro-structure part of the transglutaminase derived from *S. cinnamomeum* IFO12852 were amplified from pPKSPTG2 or pPKSPTG3 using PCR method, respectively.

[0101] The primer shown in SEQ ID NO: 48 also comprises the sequence encoding the N-terminal amino acid sequence of the mature transglutaminase derived from *S. mobaraense* IFO13819 in order to construct the fusion gene which contained the 5'-upstream region containing the promoter region of the PS2 gene of *C. glutamicum* ATCC13869, the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* and the gene for the mature transglutaminase derived from *S. mobaraense* IFO13819 having the pro-structure part of the transglutaminase derived from *Streptovercillium cinnamomeum* IFO 12852

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG3'

(SEQ ID NO: 48) 5'-GGG GTG ACC CTG TCG TCG GAG TCG GGG GCC CGG  
GAG GGC GCG CTG G-3'

<sequence listing free text>

SEQ ID NO: 48 : PCR primer

[0102] On the other hand, primers shown in SEQ ID NO: 8 and SEQ ID NO: 9 were synthesized based on the sequence of the transglutaminase gene derived from *S. mobaraense* determined in Example 1(1) and the gene region of the mature transglutaminase derived from *S. mobaraense* was amplified using PCR method from pUITG which had been obtained in Example 1(1).

(SEQ ID NO: 8) 5'-GACTCCGACGACAGGGTCACCCCTCCCGCC-3'

(SEQ ID NO: 9) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

<sequence listing free text>

SEQ ID NO:8 and 9: PCR primers

[0103] Then the fragment for the fusion gene of the mature transglutaminase gene derived from *S. mobaraense* IFO13819, which had the 5'-upstream region containing the promoter region of PS2 gene of *C. glutamicum* ATCC13869, the signal sequence of the cell surface protein of *C. ammoniagenes* and the pro-structure part of the protransglutaminase derived from *Streptovorticillium cinnamoneum* IF12852, was amplified using cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using 1 µl of PCR solution of each amplified region comprising the 5'-upstream region containing the promoter region of the PS2 gene of *C. glutamicum* ATCC13869, the region encoding the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* and the sequence encoding the pro-structure part of the transglutaminase derived from *S. cinnamoneum* IFO12852, and also 1 µl of PCR solution of the amplified gene region encoding the mature transglutaminase derived from *S. mobaraense* IFO13819, as the template. The amplified fragment of about 1.8kb was detected by agarose electrophoresis. The fragment of about 800bp, which was generated by digesting this fragment with *Sca*I and *Eco*065I, was recovered from agarose gel. The fragment excised from pKSPTG1, which had been constructed in Example 4(2), by the digestion with *Sca*I and *Eco*065I was replaced by this fragment to construct pPKSPTG4 and pPKSPTG5.

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 9) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

(2) Secretion of the transglutaminase derived from *S. mobaraense* IFO13819 using the signal sequence of the cell surface protein of *C. ammoniagenes* and the pro-structure part derived from *S. cinnamoneum* IFO12852

[0104] *C. glutamicum* ATCC13869 was transformed with the constructed plasmids pPKSPTG4 or pPKSPTG5, and the strains which grew on the CM2S agar medium described above comprising 25 mg/l kanamycin were selected. Then the selected *C. glutamicum* ATCC13869 harboring pPKSPTG4 or pPKSPTG5 was cultured in MMTG culture medium, described above, comprising 25 mg/l kanamycin at 30°C for 30 days, respectively. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot analysis was performed with anti-transglutaminase antibody as described previously according to the conventional method. Consequently the secretion of the transglutaminase derived from *S. mobaraense* IFO13819 having the *S. cinnamoneum* IFO12582 derived pro-structure part was confirmed. Table 3 shows the amount of the production of pro-transglutaminase. pPKSPTG1 was used as a control and was genetically characterized in that the pro-part was derived from *S. mobaraense*. pPKSPTG4 was characterized in the genetic construction in that the pro-part was derived from *S. cinnamoneum*. pPKSPTG5 was characterized in the genetic construction in that the pro-structure had a chimeric pro-

structure wherein the N-terminal 16 amino acids of the pro-structure part were derived from *S. mobaraense* and the C-terminal 40 amino acids was derived from *S. cinnamomeum*. Otherwise, the three had the common features. As a result, the significant difference in the amount of secretion was observed due to the difference of the amino acid sequence of the pro-structure part. The strain having the chimeric structure secreted the greatest amount of transglutaminase (ATCC13869/pPKSPTG5).

Table 3:

Amount of the secretory production of protransglutaminase by the difference of the pro-structure part	
plasmid	protransglutaminase mg/l
pPKSPTG1	235 mg/l
pPKSPTG4	130
pPKSPTG5	270

#### Example 8: Cloning of the serine protease (SAMP45) gene, and the generation and evaluation of expression plasmids

(1) Construction of the serine protease (SAMP45) gene having the pro-structure part and the signal sequence of the cell surface protein of *C. ammoniagenes* (heterologously fused prepro-serine protease (SAMP45) gene)

[0105] The sequence of the gene of SAMP45 which is a serine protease produced by *S. albogriseolus* [J. Bacteriol., 179, 430-438(1997)] has been already determined. Primers shown in SEQ ID NO: 49 and SEQ ID NO: 50 were synthesized on referring to this sequence and the gene region comprising the N-terminal pro-structure part of SAMP45, mature SAMP45 and the C-terminal pro-structure part was amplified using PCR method according to the method described previously.

(SEQ ID NO: 49) 5'-AACGGGGAGAACAGCACGGCCGCGCGG-3'

(SEQ ID NO: 50) 5'-GGCGAATTCTCCGGCGGGCCGTCACCGGT-3'

<sequence listing free text>

SEQ ID NO: 49 and SEQ ID NO: 50: PCR primers

[0106] The region comprising 5'-upstream region containing the promoter region of the gene of the cell surface protein PS2 from *C. glutamicum* and the signal sequence of the cell surface protein StpA from *C. ammoniagenes* was similarly amplified using PCR method with the combination of SEQ ID NO: 51 and SEQ ID NO: 52 with pPKSPTG1 constructed in Example 4(2) as the template.

[0107] The primer shown in SEQ ID NO: 52 comprises the sequence encoding the N-terminal amino acids of pro-serine protease in order to construct the fusion gene containing the serine protease having the pro-structure part.

(SEQ ID NO: 51) 5'-GGCAAGCTTAAATTCCTGTGAATTAGCTGA-3'

(SEQ ID NO: 52)

5'-CGGCCGTGCTGTTCTCCCCGTTTGCCGTTGCCACAGGTGCGGCC-3'

<sequence listing free text >

SEQ ID NO: 51 and SEQ ID NO: 52: PCR primers to construct the fused pro-serine protease gene

[0108] Then the gene fragment of the heterologously fused prepro-serine protease gene, which was ligated to the

signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and to the 5'-upstream region containing the promoter region of PS2 gene, was amplified by performing cross-over PCR with SEQ ID NO: 51 and SEQ ID NO: 50 using the mixture as the templates comprising 1 µl of PCR solution of the amplified region comprising the gene for the N-terminal pro-structure of SAMP45, mature SAMP45 and the C-terminal pro-structure, and 1 µl of PCR solution of the amplified region comprising the 5'-upstream region containing the promoter region of the PS2 gene and the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*, respectively.

[0109] The amplified fragment of about 3.9 kb was detected by agarose electrophoresis.

[0110] The PCR product was digested with HindIII and EcoRI, then subjected to agarose gel electrophoresis, and the fragment of about 3.9kb was recovered from agarose gel and inserted into HindIII-EcoRI site of the forementioned pVC7 to obtain pVSS1, respectively. The sequence of the inserted fragment was determined according to the forementioned method and it was confirmed that the fusion gene was constructed as expected.

## (2) Secretion of the serine protease using the signal sequence of the cell surface protein of *C. ammoniagenes*

[0111] *C. glutamicum* ATCC13869 was transformed using the plasmid pVSS1 and the strains which grew on the CM2S agar medium described above comprising 5 mg/l chloramphenicol were selected. The selected *C. glutamicum* ATCC13869 harboring pVSS1 was then cultured in MMTG culture medium comprising 5 mg/l chloramphenicol at 30°C for 70 hours. 1ml of the culture medium was separate into the supernatant of the culture medium and the cells by centrifugation. The cell was suspended in 0.1 M sodium phosphate buffer ( pH 7.0). The activity of the serine protease was determined as follows: 50µl of the supernatant of the culture medium or the cell suspension was added to 20 mM sodium phosphate buffer (pH 7.0) containing 0.25 mM Bz-Phe-Val-Arg-pNA ( Bachem Co. Ltd.) to give a total amount of 0.6 ml, which was maintained at 30°C for 20 minutes. Thereafter the reaction was terminated upon the addition of 0.4 ml of 50% acetic acid. The absorbance was measured at 410 nm and the amount of p-NA (p-nitroanillide) released was measured to determine the activity. One unit of the enzyme was defined as the amount of enzyme which releases 1 µmol of pNA per one minute. As a result, the activity of serine protease was not detected in the supernatant of the culture medium, but was detected in the cell suspension. Calculating from the values of detected activity and the values of the specific activity reported in the literature [J. Bacteriol., 179, 430-438(1997)], as much as about 9 mg/l of serine protease was confirmed to be expressed and secreted at the surface of the cell.

## (3) Cleavage of the pro-structure part of the transglutaminase having the pro-structure part by serine protease which is secreto-produced in *C. glutamicum* ATCC13869

[0112] *C. glutamicum* ATCC13869 harboring the secretory expression plasmid pPKSPTG1 for the transglutaminase having the pro-structure part described in Example 4(2) was transformed with the constructed plasmid pVSS1 and the strains grown on the CM2S agar medium, described above, comprising 5 mg/l of chloramphenicol and 25 mg/l kanamycin were selected. Next the selected *C. glutamicum* ATCC13869 harboring pVSS1 and pPKSPTG1 was cultured in the MMTG culture medium, described above, comprising 5 mg/l chloramphenicol and 25 mg/l kanamycin at 30°C for 70 hours. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot analysis was performed with anti-transglutaminase antibody previously described according to the conventional method. As a result, it was confirmed that SAMP45 was normally expressed and secreted, and that the pro-structure part was cleaved from the transglutaminase having the pro-structure part which is also secreted, resulting in the secretion of the transglutaminase having the similar molecular weight to that of the naturally occurring mature transglutaminase.

[0113] The supernatant of the culture medium was investigated for transglutaminase activity by hydroxamate method previously described, which confirmed that it had the similar specific activity (about 20 U/mg) as that of the naturally occurring transglutaminase.

[0114] Further it was semi-dry blotted on polyvinylidene-difluoride (PVDF) membrane according to the method previously described after SDS-PAGE. After blotting, the PVDF membrane was stained with Coomassie Brilliant Blue, de-stained and air-dried. The portion containing the mature transglutaminase was excised and was analyzed for the N-terminal amino acid sequence using a protein sequencer. As a result, it was confirmed that it had the structure in which the 4 C-terminal amino acids of Phe-Arg-Ala-Pro from the pro-structure part was added to the naturally occurring mature transglutaminase derived from *S. mobaraense* shown in SEQ ID NO: 5.

Example 9: Cloning of the proline specific peptidase (svPEP) gene, and the generation and evaluation of expression plasmids

(1) Purification of the proline specific peptidase (svPEP) produced by *S. mobaraense* IFO13819

[0115] 800 mL of ISP2 liquid culture medium (4 g of yeast extract, 10 g of malt extract, 4 g of glucose filled up to 1L by water, adjusted to pH 7.3) was placed in a 5L Sakaguchi flask and *S. mobaraense* IFO13819 was inoculated from the plate into the flask and cultured by shaking at 30°C for 48 hours at 120 rpm.

[0116] The culture medium was centrifuged to remove the supernatant of the culture and the cells were harvested. After washing the cells with 20 mM Tris-HCl buffer containing 25 mg/l kanamycin, the resulting cells was suspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 25 mg/l kanamycin. The suspension was shaken on ice for 4 hours and centrifuged to give the supernatant, which was collected. After the supernatant was filter-sterilized using nitrocellulose filter (0.22µm poresized, Sartorius Co. Ltd.), the supernatant was passed through the Butyl-Sepharose 4FF (Amersham Pharmacia Co. Ltd.) column (1.6φ×10 cm), which had been pre-equilibrated with 1.5 M ammonium sulfate/ 50 mM phosphate buffer (pH 7.0), using FPLC (Amersham Pharmacia Co. Ltd.) and eluted by the linear gradient of ammonium sulfate 1.5 to 0 M in the same buffer. Fractions containing active components were pooled and passed through Phenyl-Sepharose HP column (1mL, Amersham Pharmacia Co. Ltd.) under the same condition, and active fractions were pooled and dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0) at 4 °C to give partially purified enzyme solution.

[0117] The total weight, total activity, specific activity, yield of proteins at each step are presented in Table 4. The enzyme activity at each step was determined according to the method by Yoshimoto et al (Tsuru and Funatsu eds., Seibutsukagaku Jikkenhou, 31 Proteolytic enzyme II, Gakkai Shuppan Center (1993), p187) as follows:

[0118] The enzyme solution was added to 20 mM of sodium phosphate buffer containing 0.25 mM of Ala-Ala-Pro-pNA (Bachem Co. Ltd.) to give a total amount of 0.6 ml, which was maintained at 30°C for 5 minutes. Thereafter the reaction was terminated upon the addition of 0.4 ml of 50% acetic acid. The absorbance was measured at 410 nm and the amount of p-NA released was calculated to determine the activity. One unit of the enzyme was defined as the amount of the enzyme which releases 1 µmol of pNA per one minute.

Table 4.

Purification of proline specific peptidase derived from <i>S. mobaraense</i>						
Purification steps	volume (ml)	total activity (units)	total protein (mg)	specific activity (unit/mg)	yield (%)	purification level (fold)
crude extract	550	308	385	0.80	100	1
Butyl-Sepharose 4FF	45.6	213	8.98	23.7	69	30
Phenyl-Sepharose HP	5.8	136	3.83	35.5	44	44

(2) Sequencing of the N-terminal amino sequence of the proline specific peptidase (svPEP) produced by *S. mobaraense* IFO13819

[0119] Partially purified enzyme solution was subjected to reversed phase chromatography for further purification. The condition of reversed phase chromatography was as follows:

HPLC device: pump : HITACHI L-6300, detector: L-4000H  
 column : PROTEIN C4 214TP5410( VYDAC Co. Ltd.)  
 elution : Elution was effected by a lineal gradient of acetonitrile 24-40 % / 0.1% trifluoroacetic acid (20 min) at room temperature  
 flow rate : 1.0 ml/min.  
 detection wavelength : 280 nm

[0120] The enzyme samples which were purified under the condition describe above were transferred onto Polyvinylidene-difluoride (PVDF) membrane using Membrane Cartridge (Perkin Elmer Co. Ltd.) and the N-terminal amino acid sequence was analyzed using gas-phase Protein Sequencer PPSQ-10 (Shimazu Seisakusho Co., Ltd.). As a result, the N-terminal 20 amino acid residues were determined, which are shown in SEQ ID NO: 53.

(SEQ ID NO: 53) Gln Ala Asp Ile Lys Asp Arg Ile leu Lys Ile Pro

1

5

10

Gly Met Lys Phe Val Glu Glu Lys

15

20

(3) Evaluation of the properties of the proline specific peptidase (svPEP) produced by *S. mobaraense* IFO13819

[0121] Proline specific peptidase (svPEP) produced by *S. mobaraense* IFO13819 was evaluated for the following properties:

(i) Substrate specificity

[0122]

(a) When a chromophore pNA -conjugated peptide were used as a substrate: Purified enzyme solution was added to 20 mM of sodium phosphate buffer (pH 6.5) containing 0.25 mmol of each of various peptides conjugated to pNA to give a total amount of 0.6 ml, which was maintained at 37°C for 5 minutes. The reaction was terminated upon the addition of 0.4 ml of 50% acetic acid. The absorbance was measured at 410 nm to determine the cleaving activity.

(b) When a chromophoric group  $\beta$ NA( $\beta$ -naphthylamido)-conjugated peptide was used as a substrate: Purified enzyme solution was added to 20 mM of sodium phosphate buffer (pH 6.5) containing 0.3 mmol of each of various peptides to give a total amount of 1.0 ml, which was maintained at 37°C for 5 minutes. The reaction was terminated upon the addition of 0.4 ml of Fast garnet GBC solution (Fast garnet GBA was dissolved in 10 % Triton X-100/1M sodium acetate (pH 4.0) to give 0.1%). The absorbance was measured at 550 nm to determine the cleaving activity.

(c) When a peptide were used as a substrate: Enzyme solution was added to the peptide solution, which was prepared to be 1 mg/ml, as a substrate and the reaction was performed at 30°C for 1 hour. The cleaving activity was confirmed using HPLC under the following condition:

Column : YMC-PACK ODS-A 4.6×150 mm (YMC)  
 Eluent : 0.1% trifluoroacetic acid(TFA)-acetonitrile  
 Flow rate : 1 ml/ min  
 Detection wavelength: UV 220 nm

[0123] As a result, it was revealed that the enzyme was the enzyme which cleaves a peptide specifically at the carboxyl terminal side of proline residue, and that it preferably recognized Ala-Ala-Pro-pNA, Phe-Arg-Ala-Xaa (SEQ ID NO: 68) (wherein Xaa represents Pro-pNA and pNA represents p-nitroanilide), Ala-Phe-Pro-pNA in this order, and that it was most specifically reactive to the peptide which had proline at the 3rd or 4th position from the N-terminal. It was also revealed that the enzyme did not act on the peptide which had proline at the 2nd or 5th position from the N-terminal (Table 5).

Table 5.

Specifity of svPEP	
Peptide substrate	relative activity
	(%)
p-pNA	0.04
DP-pNA	0.00
Z-GP- $\beta$ NA	0.04
GP- $\beta$ NA	0.40
AP- pNA	0.53



Table 5. (continued)

Specifity of svPEP	
Peptide substrate	relative activity
RP-pNA	0.94
Z-AGP-βNA	0.78
Z-GAP-βNA	1.2
Bz-FVR-pNA	0.002
AAF-pNA	4.1
AAA-pNA	8.5
AFP-pNA	26.3
AAP-pNA	100
AAPL-pNA	0.3
FRAP-pNA	49.0
Suc-AAPF-pNA	0.01
SFRAP-pNA	1.23
PSFRAP-pNA	0.2
pNA: p-nitroanilide, βNA: β-naphtylamido	

<sequence listing free text>

SEQ ID NO: 68: substrate for svPEP

(ii) Optimum pH

[0124]

pH 4 to 6: 20 mM sodium phosphate buffer,  
pH 5.5 to 8: 20 mM sodium phosphate buffer, and  
pH 6.5 to 9.5: 20 mM tris-hydrochloride buffer

were used as a buffer, respectively. The enzyme was allowed to act on Ala-Ala-Pro-pNA as the substrate at 30°C for 5 minutes. Relative activity of the enzyme in each buffer was calculated compared to the activity in 20 mM sodium phosphate buffer, pH6.5 as 100 %. As a result, it was revealed that its optimum pH ranged from 6 to 6.5.

(iii) pH stability

[0125] 0.15 M GTA buffers (buffers composed of 3,3-dimethylglutaric acid, tris(hydroxymethyl)aminomethane, 2-amino-2-methyl-1,3-propanediol) ranging between pH 3 and pH10 were used. To 20 μl of purified enzyme solution, 40 μl of the buffer at each pH was added and the reaction mixtures were allowed to stand overnight at 4°C, then adjusted to pH 7.0 and the volume was made up to 120 μl. To 50 μl aliquots, Ala-Ala-Pro-pNA was added and the reaction was performed at 30 °C for 5 minutes. Relative amounts of substrate which the enzyme decomposed at each pH were considered to be the remaining activity compared to the activity of the enzyme as 100% when the enzyme was stored under the same condition as described above except for pH 7.0. As a result, it was revealed that the enzyme was stable at pH 4 to 9.

(iv) Optimum temperature

[0126] To 50 μl of purified enzyme solution, 0.5 ml of 20 mM sodium phosphate buffer (pH 6.5) was added and Ala-Ala-Pro-pNA was added up to 0.25 mM and the mixture was maintained at 20°C-60°C for 5 minutes to effect decomposition reaction. Relative amount of substrate which the enzyme decomposed was considered to be the relative

activity at each temperature, compared to the amount of substrate decomposition at 25°C as 100% activity. As a result, it was revealed that its optimum temperature ranged from 25 to 30 °C.

(v) Temperature stability

[0127] To 50 µl of purified enzyme solution, 0.5 ml of 20 mM sodium phosphate buffer(pH 6.5) was added. The mixture was maintained at 4 °C or 20°C-60°C for 15 minutes and then cooled on ice. Ala-Ala-Pro-pNA was added up to 0.25 mM and the mixture was maintained at 30°C for 5 minutes. The remaining activity was calculated assuming the activity of the enzyme which was treated at 4°C to be 100%. As a result, it was revealed that the enzyme was stable below 20°C.

(vi) Inhibitors

[0128] To 20 mM sodium phosphate buffer (pH 6.5) containing each compound at the indicated concentration shown in Table 6, purified enzyme solution was added and the mixture was allowed to stand at room temperature for 10 minutes. Then Ala-Ala-Pro-pNA was added and the reaction was performed at 30°C for 5 minutes. Assuming the activity of the enzyme to Ala-Ala-Pro-pNA in the absence of compounds to be 100%, relative amount of substrate decomposition in the presence of the compound was considered to be the relative activity. As a result, the enzyme was inhibited to some extent by chloromercuribenzoic acid, etc. which is the SH-enzyme inhibitors, but it was undergone the comparatively strong inhibition by phenylmethylsulfonylfluoride ( Nakaraites Co., Ltd.) and aminoethylbenzenesulfonylfluoride hydrochloride ( Boeringer Mannheim Co., Ltd.) which were the serine protease inhibitors.

Table 6.

Effects of inhibitors to the activity of proline specific peptidase derived from <i>S. mobaraense</i>		
Compounds	Concentration (mM)	relative activity(%)
None	0	100
Serine enzyme inhibitors		
Phenylmethylsulfonylfluoride	1	39.7
Aminoethylbenzenesulfonylfluoride hydrochloride	4	59.9
Chymostatin	1	84.9
SH-enzyme inhibitors		
p-Chloromercuribenzoic acid	1	87.1
N-Ethylmaleimide	1	98.3
Iodoacetamide	1	87
Asparagine enzyme inhibitor		
Pepstatin	1	165.7
Metalloprotease inhibitors		
EDTA	10	105.2
1,10-Phenanthroline	1	92.5
Aminopeptidase inhibitor		
Bestatin	1	97.6
Reducing agent		
Dithiothreitol	10	102.5
Prolylendopetidase inhibitors		
Z-(S)Pro-(S)Prolinal	1	111.2
Z-Pro-(S)Prolinal	1	105.8
Z-Pro-Prolinal	1	99.7

(3) Acquisition of the proline specific peptidase(svPEP) gene derived from *S. mobaraense* IFO13819

[0129] The region having less degeneracy which is deduced from the determined N-terminal amino acid sequence of svPEP, Lys-Ile-Pro-Gly-Met-Lys-Phe-Val-Glu-Glu-Lys, was selected and the synthetic oligonucleotide shown in SEQ

ID NO: 54 was generated. The chromosomal DNA prepared according to the conventional method was digested with various restriction enzymes which recognize 6-nucleotides sequence and then analyzed by Southern blot hybridization method using this synthetic oligonucleotide as the probe and thereby a single band of about 6 kb was detected by SacI cleavage. Accordingly, the chromosomal DNA of *S. mobaraense* IFO13819 prepared according to the forementioned method was digested with Sac I and the fragment of about 6 kb was recovered using agarose gel electrophoresis with EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.). The recovered fragment was inserted in Sac I site of pUC18, which was introduced into the competent cell of *Escherichia coli* JM109 (Takarashuzo Co. Ltd.), thereby producing a library. The generated library in this way was screened for the strain which harbored the plasmid wherein the fragment of svPEP gene was cloned, by screening the library through colony hybridization using <sup>32</sup>P-labelled synthetic oligonucleotide shown in SEQ ID NO: 54 as a probe to obtain the intended gene. The plasmid recovered from this strain was designated as pUMP1.

(SEQ ID NO: 54) 5'-AAGATCCCCGGGATGAAGTTCGTGAGGAGAAG-3'

<sequence listing free text>

SEQ ID NO: 54 : a probe for svPEP

[0130] The nucleotide sequence of the fragment which was cloned as pUMP1 was determined. The nucleotide sequence of svPEP gene corresponding to svPEP is shown in SEQ ID NO: 41. The amino acid sequence encoded by this gene was deduced and the previously determined N-terminal amino acid sequence (20 residues) based on the enzyme protein was found, and the primary amino acid sequence of mature svPEP shown in SEQ ID NO:40 was determined. The entire primary amino acid sequence containing the putative signal sequence and the pro-structure part of svPEP was determined, which is shown in SEQ ID NO: 42.

[0131] *Escherichia coli* AJ13669 which was transformed with pUMP1 has been deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology on May 15, 2000 as FERM BP-7160 under the Budapest Treaty.

(4) Construction of the proline specific peptidase (svPEP) gene having the pro-structure part with the signal sequence of the cell surface protein of *C. ammoniagenes* (heterologously fused prepro-proline specific peptidase (svPEP) gene)

[0132] Primers shown in SEQ ID NO: 55 and SEQ ID NO: 56 were synthesized on referring to the sequence of svPEP determined in Example 9(3), and the gene region containing the pro-part of svPEP and mature svPEP were amplified by PCR method in the same manner as described previously using pUMP1 constructed in Example 9(3) as the template.

(SEQ ID NO: 55) 5'-GAGGCGGCGTCGATCACCGCCCC-3'

(SEQ ID NO: 56) 5'-GCCAAGCTTGAAGCACCGGGCGGCGGCACCCGG-3'

<sequence listing free text>

SEQ ID NO: 55 and SEQ ID NO: 56: PCR primers

[0133] Then the region, which comprises the 5'-upstream region containing the promoter region of PS2 gene which is the gene of the cell surface protein of *C. glutamicum* and the region containing the signal sequence of the cell surface protein StpA of *C. ammoniagenes*, was amplified by PCR method from pPKSPTG1 constructed in Example 4(2) as the template using the combination of SEQ ID NO: 51 and SEQ ID NO: 57.

[0134] The primer shown in SEQ ID NO: 57 comprises the sequence encoding the N-terminal amino acids of svPEP in order to construct the fusion gene fused to the svPEP having the pro-structure part.

(SEQ ID NO: 51) 5'-GGCAAGCTTAAATTCCTGTGAATTAGGCTGA-3'

(SEQ ID NO: 57)

5'-GGGGCGGTGATCGACGCCGCCTCTGCCGTTGCCACAGGTGCGGCCA-3'

&lt;sequence listing free text&gt;

SEQ ID NO: 57 : PCR primer

[0135] The fragment of the heterologously fused gene of prepro-svPEP, which was ligated to the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and the 5'-upstream region containing the promoter region of PS2 gene, was then amplified by performing cross-over PCR with SEQ ID NO: 51 and SEQ ID NO: 56 using the mixture as the templates comprising 1 µl of each PCR solution of the region containing the gene encoding the pro-structure part of svPEP and the mature svPEP, which were amplified respectively, and 1 µl of PCR solution of the amplified region comprising 5'-upstream region containing the promoter region of the PS2 gene and the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*.

(SEQ ID NO: 51) 5'-GGCAAGCTTAAATTCCTGTGAATTAGCTTA-3'

(SEQ ID NO: 56) 5'-GCCAAGCTTGAAGCACCGGCGGGCGGCACCCGG-3'

[0136] The amplified fragment of about 2.1 kb was detected by agarose electrophoresis.

[0137] The PCR fragment was digested with HindIII, and then subjected to agarose gel electrophoresis and the fragment of about 2.1 kb recovered from the agarose gel and inserted into the HindIII site of the pVSS1 described in Example 8(1) to obtain pVSSSP1, respectively. The sequence of the inserted fragment was determined according to the conventional method and it was confirmed that the expected fusion gene was constructed.

(5) Secretion of the proline specific peptidase using the signal sequence of the cell surface protein of *C. ammoniagenes*

[0138] *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVSSSP1 and the strains which grew on the CM2S agar medium described above comprising 5 mg/l chloramphenicol were selected. The selected *C. glutamicum* ATCC13869 harboring pVSSSP1 was then cultured in MMTG culture medium, described above, comprising 5 mg/l chloramphenicol at 30°C for 70 hours. 10 µl of the supernatant of the culture was separated by centrifugation into the supernatant of the culture medium and the cells. The cells were suspended in 0.1 M sodium phosphate buffer (pH 7.0). The activity of svPEP was determined as follows: 50 µl of the supernatant of the culture medium or the cell suspension was added to 20 mM sodium phosphate buffer (pH 7.0) containing 0.25 mM Ala-Ala-Pro-pNA (Bachem Co. Ltd.) to give a total amount of 0.6 ml and the mixture was maintained at 30°C for 20 minutes. Thereafter the reaction was terminated upon the addition of 0.4 ml of 50% acetic acid. The absorbance was measured at 410 nm and the amount of p-NA (p-nitroanillide) released was calculated to determine the activity. One unit of the enzyme is defined as the amount of enzyme which releases 1 µmol of pNA per 1 minute. As a result the activity of svPEP was not detected in the supernatant of the culture medium, but was detected in the cell suspension. Calculating from the values of the detected activity and the values of the specific activity described in Example 9(1), as much as about 50 mg/l of svPEP was confirmed to be expressed and secreted at the surface of the cell.

(6) Cleavage of the pro-structure part by the serine protease and proline specific protease expressed and secreted by *C. glutamicum* ATCC13869

[0139] *C. glutamicum* ATCC13869 harboring the secretory expression plasmid pPKSPTG1 for transglutaminase having the pro-structure part described in Example 4(2) was transformed with the constructed plasmid pVSSSP1, and the strains grown on the CM2S agar medium, described above, comprising 5 mg/l of chloramphenicol and 25 mg/l kanamycin were selected. Then the selected *C. glutamicum* ATCC13869 harboring pVSSSP1 and pPKSPTG1 was cultured in MMTG culture medium, described above, comprising 5 mg/l chloramphenicol and 25 mg/l kanamycin at 30°C for 70 hours. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and

then Western blot analysis was performed with anti-transglutaminase antibody previously described according to the conventional method. As a result, it was confirmed that SAMP45 and svPEP were normally expressed and secreted, and that the pro-structure part was cleaved from the transglutaminase having the pro-structure part which had been also secreted, thereby the secretion of the transglutaminase having the similar molecular weight to that of the naturally occurring mature transglutaminase was confirmed.

[0140] The transglutaminase activity was tested for the supernatant by the hydroxamate method previously described, which confirmed that it contained the similar specific activity (about 20 U/mg) to that of the naturally occurring transglutaminase.

[0141] Further it was semi-dry blotted onto polyvinylidene difluoride (PVDF) membrane according to the method previously described after SDS-PAGE. After blotting, the PVDF membrane was stained with Coomassie Brilliant Blue, de-stained and air-dried. The portion containing the mature transglutaminase was removed and analyzed for the N-terminal amino acid sequence using a protein sequencer. As a result, it was confirmed that it had the same sequence as the naturally occurring transglutaminase derived from *S. mobaraense* having Asp as the N-terminal amino acid, which is shown in SEQ ID NO: 5.

#### Example 10: Generation of the partial deletion variant of the pro-structure of *S. mobaraense* IFO13819 derived pro-transglutaminase and the secretory production of transglutaminase

##### (1) Construction of the gene for partial deletion variants of the pro-structure of the transglutaminase

[0142] In order to generate the partial deletion form wherein the C-terminal amino acid residues of the pro-structure part were deleted, primers shown in SEQ ID NO: 8 and SEQ ID NO: 9 were synthesized based on the sequence of the transglutaminase gene determined in Example 1(1) and the gene region of the mature transglutaminase was amplified using pUITG obtained in Example 1(1) by the similar PCR method as described previously.

[0143] Then the gene region comprising the 5'-upstream region containing the promoter of the PS2 gene which was the cell surface protein of *C. glutamicum*, the region of the gene for the signal sequence of cell surface protein StpA of *C. glutamicum* and the pro-structure part of transglutaminase was amplified with the combination of SEQ ID NO: 14 and SEQ ID NO: 58 or of SEQ ID NO: 14 and SEQ ID NO: 59 using pKSPTG1 constructed in Example 4(2) as the template.

[0144] The primer shown in SEQ ID NO: 58 has the sequence which is defective of two C-terminal amino acid residues, Ala-Pro, of the pro-structure part of transglutaminase, and the primer shown in SEQ ID NO: 59 has the sequence which is defective of the C-terminal 4 amino acid residues, Phe-Arg-Ala-Pro, of the pro-structure part of the transglutaminase and further comprises the sequence encoding the N-terminal amino acid residues of the mature transglutaminase in order to construct the fusion gene with mature transglutaminase.

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 58) 5'-GTG ACC CTG TCG TCG GAG TCC CGG AAC GAC GGG  
CCG GCG C-3'

(SEQ ID NO: 59) 5'-GTG ACC CTG TCG TCG GAG TCC GAC GGG CCG GCG  
CTC GAA G-3'

<sequence listing free text>

SEQ ID NO: 58 and SEQ ID NO: 59: PCR primers

[0145] The gene fragment for the mature transglutaminase, which was ligated to the deletion form of the pro-structure of the transglutaminase, the signal sequence of the cell surface protein StpA of *C. ammoniagenes* and the 5'-upstream

region containing the promoter region of the gene of PS2 which is the cell surface protein of *C. glutamicum*, was amplified respectively using cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using as the template 1 µl of each PCR solution comprising the amplified 5'-upstream region containing the promoter region of PS2 gene which is the cell surface protein of *C. glutamicum*, the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and each of the modified pro-structure part, and 1 µl of PCR solution of the amplified region encoding the mature transglutaminase.

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 9) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

[0146] The amplified fragment of about 1.8 kb was detected by agarose electrophoresis.

[0147] The fragment of about 800 bp which was generated by digesting this fragment with restriction enzymes *ScaI* and *Eco065I* was recovered from agarose gel and was replaced for the fragment cleaved from pPKSPTG1, which was constructed in Example 4(2), with *ScaI* and *Eco065I* to construct pPKSPTG1ΔAP (Ala-Pro deletion type) and pPKSPTG1ΔFRAP (Phe-Arg-Ala-Pro deletion type).

[0148] Then in order to generate the partial deletion form of the pro-part wherein the N-terminal amino acid residues of the pro-structure part were partially deleted, primers shown in SEQ ID NO: 60 and SEQ ID NO: 61 were synthesized based on the sequence of the gene of the transglutaminase determined in Example 1(1) and the regions of the mature transglutaminase gene were amplified using pUITG obtained in Example 1(1) by the same PCR with the combination of SEQ ID NO: 60 and SEQ ID NO: 9 or of SEQ ID NO: 61 and SEQ ID NO: 9.

(SEQ ID NO: 60) 5'-AAT GGC GCG GGG GAA GAG ACG AAG TCC TAC GCC  
GAA ACC T-3'

(SEQ ID NO: 61) 5'-GAG ACG AAG TCC TAC GCC GAA ACC TAC CGC CTC  
ACG GCG G-3'

(SEQ ID NO: 9) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

<sequence listing free text>

SEQ ID NO: 60 and SEQ ID NO: 61 : PCR primers

[0149] Then the regions comprising the 5'-upstream region containing the promoter of the gene of PS2 which was the cell surface protein of *C. glutamicum* and the region for the signal sequence of cell surface protein SlpA of *C. glutamicum* were amplified with the combination of SEQ ID NO: 14 and SEQ ID NO: 62 or of SEQ ID NO: 14 and SEQ ID NO: 63 using pPKSPTG1 constructed in Example 4(2) as the template.

[0150] The primer shown in SEQ ID NO: 62 has the sequence defective of the N-terminal first amino acid residue, Asp, of the pro-part of the transglutaminase, the primer shown in SEQ ID NO: 63 has the sequence defective of the N-terminal 6 amino acid residues, Asp-Asn-Gly-Ala-Gly-Glu, and further they comprise the sequence encoding the C-terminal amino acid residues of the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* in order to construct the fusion gene fused with the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*.

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 62) 5'-GTC TCT TCC CCC GCG CCA TTT GCC GTT GCC ACA GGT  
GCG G-3'

(SEQ ID NO: 63) 5'-TCG GCG TAG GAC TTC GTC TCT GCC GTT GCC ACA GGT  
GCG G-3'

<sequence listing free text>

SEQ ID NO: 62 and SEQ ID NO: 63: PCR primers

**[0151]** The fragment of the mature transglutaminase gene, which is ligated to the partial deletion type for the pro-structural part of the transglutaminase, to the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and also to the 5'-upstream region containing the promoter region of the gene of PS2 which is the cell surface protein of *C. glutamicum*, was amplified respectively using cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using 1 µl of PCR solution for the 5'-upstream region containing the promoter region of the gene of PS2 which is the cell surface protein of *C. glutamicum* and the region encoding the region comprising the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and 1 µl of PCR solution of the amplified region encoding the pro-transglutaminase wherein the N-terminal of the pro-structure part was partially deleted, as the template respectively.

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 9) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

**[0152]** The amplified fragments of about 1.8 kb were detected by agarose electrophoresis. The fragments of about 800 bp which were generated by digesting these fragments with restriction enzymes *ScaI* and *Eco65I* were recovered from agarose gel and were replaced for the fragment excised from pPKSPTG1 with *ScaI* and *Eco65I*, which was constructed in Example 4(2), to construct pPKSPTG1ΔD (Asp deletion type) and pPKSPTG1ΔDNGAGE (Asp-Asn-Gly-Ala-Gly-Glu deletion type).

(2) Secretion of the transglutaminase having the partial deletion form of the pro-structure

**[0153]** *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pPKSPTG1ΔAP, pPKSPTG1ΔFRAP, pPKSPTG1ΔD or pPKSPTG1ΔDNGAGE and the strains grown on the CM2S agar medium, described above, comprising 25 mg/l kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pPKSPTG1ΔAP, pPKSPTG1ΔFRAP, pPKSPTG1ΔD or pPKSPTG1ΔDNGAGE was then cultured in MMTG culture medium, described above, comprising 25 mg/l kanamycin at 30°C for 48 hours, respectively. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot analysis was performed with anti-transglutaminase antibody previously described according to the conventional method. As a result, the secretion of the transglutaminase wherein the pro-structure part was partially deleted was confirmed. The transformant harboring pPKSPTG1ΔAP, pPKSPTG1ΔFRAP or pPKSPTG1ΔD showed the secretion equivalent to that of the naturally occurring form (pPKSPTG1), respectively, but the transformant harboring pPKSPTG1ΔDNGAGE showed about a half secretion relative to that of the naturally occurring form (pPKSPTG1).



(3) Cleavage of the pro-structure part of the protransglutaminase having the partial deletion form of the pro-structure by serine protease secreto-produced by *C. glutamicum* ATCC13869

[0154] *C. glutamicum* ATCC13869 harboring the secretory expression plasmid pPKSPTG1ΔAP, pPKSPTG1ΔFRAP, pPKSPTG1ΔD or pPKSPTG1ΔDNGAGE for the protransglutaminase having the partial deletion form of the pro-structure, which were described in Example 10 (2), was transformed with the plasmid pVSS1 constructed in Example 8(1) and the strains grown on the CM2S agar medium, described above, comprising 5 mg/l of chloramphenicol and 25 mg/l kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pVSS1 and pPKSPTG1ΔAP, pPKSPTG1ΔFRAP, pPKSPTG1ΔD or pPKSPTG1ΔDNGAGE was then cultured in the MMTG liquid culture medium, described above, comprising 5 mg/l chloramphenicol and 25 mg/l kanamycin at 30°C for 70 hours. After the incubation was finished, 10 μl of the supernatant of the culture was subjected to SDS-PAGE and then western-blotted with anti-transglutaminase antibody previously described according to the conventional method.

[0155] As a result, SAMP45 was confirmed to be normally expressed and secreted, and that the pro-structure part of the similarly secreted pro-transglutaminase having the partial deletion form of the pro-structure was cleaved, resulting in the secretion of the transglutaminase having the similar molecular weight to that of the naturally occurring mature transglutaminase.

[0156] Further semi-dry blotted onto PVDF membrane was performed after SDS-PAGE, according to the same method as previously described. After the blotting, the PVDF membrane was stained with Coomassie Brilliant Blue, destained and air-dried. The portions containing the mature transglutaminase were removed and were analyzed for the N-terminal amino acid sequence using a protein sequencer. As a result, it was confirmed that Phe-Arg was added to the N-terminal of the naturally occurring mature transglutaminase shown in SEQ ID NO: 5 in the transformant harboring pPKSPTG1ΔAP, Ser-Ala-Gly-Pro-Ser was added to the N-terminal of the naturally occurring mature transglutaminase in the transformant harboring pPKSPTG1ΔFRAP, and that Phe-Arg-Ala-Pro was added to the naturally occurring mature transglutaminase in the transformant harboring pPKSPTG1ΔD or pPKSPTG1ΔDNGAGE.

#### Example 11: Generation of the variants for pro-structure part of transglutaminase derived from *S. mobaraense* IFO13819 and secretory production of transglutaminase

(1) Construction of the pro-transglutaminase genes having the variants for pro-structure part

[0157] Primers shown in SEQ ID NO: 8 and SEQ ID NO: 9 were synthesized based on the sequence of the gene of the transglutaminase determined in Example 1(1) and the region of the gene of mature transglutaminase was amplified from pUITG obtained in Example 1(1) using the PCR method.

[0158] Then the region, which comprises the 5'-upstream region containing the promoter of the gene of PS2 which was the cell surface protein of *C. glutamicum*, the region of the gene of the signal sequence of cell surface protein SlpA of *C. glutamicum* and the pro-structure part of transglutaminase, was amplified using PCR method with the combination of SEQ ID NO: 14 and SEQ ID NO: 64, or of SEQ ID NO: 14 and SEQ ID NO: 65, or of SEQ ID NO: 14 and SEQ ID NO: 66, or of SEQ ID NO: 14 and SEQ ID NO: 67 using pPKSPTG1 constructed in Example 4(2) as the template.

[0159] The primer shown in SEQ ID NO: 64 comprises the gene sequence wherein the C-terminal 3 amino acid residues of the pro-structure part of transglutaminase, Arg-Ala-Pro were converted into Gly-Pro-Lys, the primer shown in SEQ ID NO: 65 comprises the gene sequence wherein the C-terminal 3 amino acid residues of the pro-structure part of transglutaminase, Arg-Ala-Pro, were converted into Gly-Pro-Arg, the primer shown in SEQ ID NO: 66 comprises the gene sequence wherein the C-terminal 5 amino acid residues of the pro-structure part of transglutaminase, Ser-Phe-Arg-Ala-Pro were converted into only Lys, and the primer shown in SEQ ID NO: 67 comprises the gene sequence wherein Ser-Phe-Arg-Ala-Pro were converted to only Arg.

[0160] Further they comprises the sequence encoding the N-terminal amino acid residues of mature transglutaminase in order to construct the fusion gene fused with mature transglutaminase

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTAG-3'

(SEQ ID NO: 64) 5'-GTG ACC CTG TCG TCG GAG TCC TGG CCG AAC GAC

GGG C-3'

(SEQ ID NO: 65) 5'-GTG ACC CTG TCG TCG GAG TCG CGG GGG CCG AAC  
GAC GGG C G-3'

(SEQ ID NO: 66) 5'- GTG ACC CTG TCG TCG GAG TCC TCC GGG CCG GCG  
CTC GAA G-3'

(SEQ ID NO: 67) 5'- GTG ACC CTG TCG TCG GAG TCG CGC GGG CCG GCG  
CTC GAA G-3'

<sequence listing free text>

[0161]

SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66 and SEQ ID NO: 67 : PCR primers

[0162] The transglutaminase gene fragments, which were ligated to the modified pro-structure part of the transglutaminase having the modified pro-structure, the signal sequence of the cell surface protein SlpASlpA of *C. ammoniagenes* and the 5'-upstream region containing the promoter region of the gene of PS2 which is the cell surface protein of *C. glutamicum*, was amplified by cross-over PCR with SEQ ID NO: 14 and SEQ ID NO: 9 using 1 µl of PCR solution of the respectively amplified region comprising the genes encoding the 5'-upstream region comprising the promoter region of the gene of PS2 which is the cell surface protein of *C. glutamicum*, the signal sequence of the cell surface protein SlpASlpA of *C. ammoniagenes*, and the region encoding the region containing the modified pro-structure part, and 1 µl of PCR solution of the amplified region encoding the mature transglutaminase.

(SEQ ID NO: 14) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 9) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

[0163] The amplified fragments of about 1.8 kb were detected by agarose electrophoresis. The fragments of about 800 bp, which were generated by digesting these fragment with restriction enzymes *ScaI* and *Eco065I*, were recovered from agarose gel and were replaced for the fragment cleaved from pPKSPTG1 constructed in Example 4(2) with *ScaI* and *Eco065I* to construct pPKSPTG11 (Gly-Pro-Lys type modified variant) and pPKSPTG12 (Gly -Pro-Arg type modified variant), pPKSPTG13 (Δphe-Arg-Ala-Pro type and Lys insertion-variant) and pPKSPTG14 (Δphe-Arg-Ala-Pro type and Arg insertion-variant).

(2) Secretion of the transglutaminase in the modified pro-structure part form

[0164] *C. glutamicum* ATCC13869 was transformed with the constructed plasmids pPKSPTG11, pPKSPTG12, pPKSPTG13 or pPKSPTG14 and the strains grown on the CM2S agar medium, described above, comprising 25 mg/l kanamycin were selected. Then the selected *C. glutamicum* ATCC13869 harboring pPKSPTG11, pPKSPTG12, pPKSPTG13 or pPKSPTG14 was cultured in MMTG culture medium, described above, comprising 25 mg/l kanamycin at 30°C for 48 hours, respectively. After the incubation was finished, 10 µl of the supernatant of the culture was subjected

to SDS-PAGE and then Western blot analysis was performed with the anti-transglutaminase antibody previously described according to the conventional method. As a result, the secretion of the transglutaminase having the pro-structure part was confirmed.

(3) Cleavage of the pro-structure part of the transglutaminase having the pro-structure part variant with serine protease produced by secretory production by *C. glutamicum* ATCC13869

[0165] *C. glutamicum* ATCC13869 harboring the secretory expression plasmid pPKSPTG11, pPKSPTG12, pPKSPTG13 or pPKSPTG14 for the transglutaminase having the pro-structure part variant, which was described in Example11(2), was transformed with the plasmid pVSS1 constructed in Example8(1) and the strains grown on the CM2S-agar medium, described above, comprising 5 mg/l of chloramphenicol and 25 mg/l kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pVSS1 and pPKSPTG11, pVSS1 and pPKSPTG12, pVSS1 and pPKSPTG13, or pVSS1 and pPKSPTG14 was then cultured in the MMTG liquid culture medium, described above, comprising 5 mg/l chloramphenicol and 25 mg/l kanamycin at 30°C for 70 hours. After the incubation was finished, 10 µl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot analysis was performed with anti-transglutaminase antibody previously described according to the conventional method. As a result, SAMP45 was confirmed to be normally expressed and secreted, the pro-structure variant of the transglutaminase having the pro-structure variant, which had been also secreted, was confirmed to be cleaved, and consequently the secretion of transglutaminase having the similar molecular weight to that of the naturally occurring mature transglutaminase was confirmed.

[0166] Further semi-dry blotting onto PVDF membrane was performed according to the same method as previously described after SDS-PAGE. After the blotting, the PVDF membrane was stained with Coomassie Brilliant Blue, destained and air-dried. The portions containing mature transglutaminase were excised and were analyzed for the N-terminal amino acid sequence using a protein sequencer. As a result, the sequence identical to that of the naturally occurring mature transglutaminase was confirmed for *C. glutamicum* ATCC13869 harboring pVSS1 and pPKSPTG11, or pVSS1 and pPKSPTG12, which had Asp as the N-terminal amino acid residue. In contrast, the sequence wherein Ser-Ala-Gly-Pro-Lys (SEQ ID NO: 69) or Ser-Ala-Gly-Pro-Arg (SEQ ID NO: 70) was found to be added to the sequence of the naturally occurring mature transglutaminase for *C. glutamicum* ATCC13869 harboring pVSS1 and pPKSPTG13, or pVSS1 and pPKSPTG14.

[0167] The transglutaminase activity was determined for the supernatant of the culture medium of the former which showed the same amino acid sequence as that of the naturally occurring mature transglutaminase, which confirmed that they had almost the same specific activity (about 20 U/mg) as that of the naturally occurring transglutaminase as determined by hydroxamate method previously described.

<sequence listing free text>

SEQ ID NO: 69, SEQ ID NO: 70 : sequence added to the sequence of the naturally occurring transglutaminase

[0168] According to the present invention it is possible to make a coryneform bacterium to produce and secrete a large amount of a useful protein, particularly transglutaminase extracellularly. Since the protein produced according to the present invention is released into the culture medium, the protein can be directly recovered from the culture medium more simply and in a larger scale by known appropriate methods.

SEQUENCE LISTING

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30

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5

10

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35 40 45

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25 Val Gly Val Thr Trp Val Asn Ser Gly Gln Tyr Pro Thr Asn Arg Leu  
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Pro Arg Ser Pro Thr Ser Pro Gly Glu Gly Phe Val Asn Phe Asp Tyr

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Gly Trp Phe Gly Ala Gln Thr Glu Ala Asp Ala Asp Lys Thr Val Trp

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Thr His Gly Asn His Tyr His Ala Pro Asn Gly Ser Leu Gly Ala Met

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His Val Tyr Glu Ser Lys Phe Arg Asn Trp Ser Glu Gly Tyr Ser Asp

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5	gac ccg gac gcc ttc cgc ccc gcc ccg ggc acc ggc ctg gtc gac atg 1507			
	Asp Pro Asp Ala Phe Arg Pro Ala Pro Gly Thr Gly Leu Val Asp Met			
10	295	300	305	310
15	tcg agg gac agg aac att ccg cgc agc ccc acc agc ccc ggt gag gga 1555			
	Ser Arg Asp Arg Asn Ile Pro Arg Ser Pro Thr Ser Pro Gly Glu Gly			
	315	320	325	
20	ttc gtc aat ttc gac tac ggc tgg ttc ggc gcc cag acg gaa gcg gac 1603			
	Phe Val Asn Phe Asp Tyr Gly Trp Phe Gly Ala Gln Thr Glu Ala Asp			
25	330	335	340	
30	gcc gac aag acc gtc tgg acc cac gga aat cac tat cac gcg ccc aat 1651			
	Ala Asp Lys Thr Val Trp Thr His Gly Asn His Tyr His Ala Pro Asn			
	345	350	355	
35	ggc agc ctg ggt gcc atg cat gtc tac gag agc aag ttc cgc aac tgg 1699			
	Gly Ser Leu Gly Ala Met His Val Tyr Glu Ser Lys Phe Arg Asn Trp			
40	360	365	370	
45	tcc gag ggt tac tcg gac ttc gac cgc gga gcc tat gtg atc acc ttc 1747			
	Ser Glu Gly Tyr Ser Asp Phe Asp Arg Gly Ala Tyr Val Ile Thr Phe			
	375	380	385	390
50	atc ccc aag agc tgg aac acc gcc ccc gac aag gta aag cag ggc tgg 1795			
	Ile Pro Lys Ser Trp Asn Thr Ala Pro Asp Lys Val Lys Gln Gly Trp			
55	395	400	405	

ccg tgatgtgagc g

Pro

<210> 13

<211> 407

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence:PCR primer

<400> 13

Met Arg Ile Arg Arg Arg Ala Leu Val Phe Ala Thr Met Ser Ala Val

1

5

10

15

Leu Cys Thr Ala Gly Phe Met Pro Ser Ala Gly Glu Ala Ala Ala Asp

20

25

30

Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg Leu

35

40

45

Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala Pro

50

55

60

Ala Ala Ser Ser Ala Gly Pro Ser Phe Arg Ala Pro Asp Ser Asp Asp

65

70

75

80

Arg Val Thr Pro Pro Ala Glu Pro Leu Asp Arg Met Pro Asp Pro Tyr

85

90

95

5 Arg Pro Ser Tyr Gly Arg Ala Glu Thr Val Val Asn Asn Tyr Ile Arg  
 100 105 110

10 Lys Trp Gln Gln Val Tyr Ser His Arg Asp Gly Arg Lys Gln Gln Met  
 115 120 125

15 Thr Glu Glu Gln Arg Glu Trp Leu Ser Tyr Gly Cys Val Gly Val Thr  
 130 135 140

20 Trp Val Asn Ser Gly Gln Tyr Pro Thr Asn Arg Leu Ala Phe Ala Ser  
 145 150 155 160

25 Phe Asp Glu Asp Arg Phe Lys Asn Glu Leu Lys Asn Gly Arg Pro Arg  
 165 170 175

30 Ser Gly Glu Thr Arg Ala Glu Phe Glu Gly Arg Val Ala Lys Glu Ser  
 180 185 190

35 Phe Asp Glu Glu Lys Gly Phe Gln Arg Ala Arg Glu Val Ala Ser Val  
 195 200 205

40 Met Asn Arg Ala Leu Glu Asn Ala His Asp Glu Ser Ala Tyr Leu Asp  
 210 215 220

45 Asn Leu Lys Lys Glu Leu Ala Asn Gly Asn Asp Ala Leu Arg Asn Glu  
 225 230 235 240

50 Asp Ala Arg Ser Pro Phe Tyr Ser Ala Leu Arg Asn Thr Pro Ser Phe

	245	250	255
5	Lys Glu Arg Asn Gly Gly Asn His Asp Pro Ser Arg Met Lys Ala Val		
	260	265	270
10	Ile Tyr Ser Lys His Phe Trp Ser Gly Gln Asp Arg Ser Ser Ser Ala		
15	275	280	285
	Asp Lys Arg Lys Tyr Gly Asp Pro Asp Ala Phe Arg Pro Ala Pro Gly		
20	290	295	300
	Thr Gly Leu Val Asp Met Ser Arg Asp Arg Asn Ile Pro Arg Ser Pro		
25	305	310	315 320
	Thr Ser Pro Gly Glu Gly Phe Val Asn Phe Asp Tyr Gly Trp Phe Gly		
30	325	330	335
	Ala Gln Thr Glu Ala Asp Ala Asp Lys Thr Val Trp Thr His Gly Asn		
35	340	345	350
	His Tyr His Ala Pro Asn Gly Ser Leu Gly Ala Met His Val Tyr Glu		
40	355	360	365
	Ser Lys Phe Arg Asn Trp Ser Glu Gly Tyr Ser Asp Phe Asp Arg Gly		
45	370	375	380
	Ala Tyr Val Ile Thr Phe Ile Pro Lys Ser Trp Asn Thr Ala Pro Asp		
50	385	390	395 400
55			

Lys Val Lys Gln Gly Trp Pro

405

5

10

<210> 14

15

<211> 26

<212> DNA

<213> Artificial Sequence

20

<220>

<223> Description of Artificial Sequence:PCR primer

25

<400> 14

aaattcctgt gaattagctg atttag

26

30

35

<210> 15

<211> 44

<212> DNA

40

<213> Artificial Sequence

<220>

45

<223> Description of Artificial Sequence:PCR primer

50

<400> 15

gagctctccg gcgtatgcgc atagaggcga aggctccttg aata

44

55

<210> 16

<211> 30

<212> DNA

<213> Artificial Sequence

$\langle 220 \rangle$

<223> Description of Artificial Sequence:PCR primer

<400> 16

atgcgcatac gccggagagc tctcgtcttc

30

<210> 17

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 17

ggggtgacc tgcgtcgga gtcgttgaag ccgttgttga tgttgaa

47

<210> 18

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

5 <223> Description of Artificial Sequence:PCR primer

<400> 18

10 cttcgtctct tccccgcgc cattgtcagc gaatgctggg atagcaacgc c 51

15 <210> 19

<211> 51

20 <212> DNA

<213> Artificial Sequence

25 <220>

<223> Description of Artificial Sequence:PCR primer

30 <400> 19

cttcgtctct tccccgcgc cattgtcctg agcgaatgct gggatagcta c 51

35 <210> 20

40 <211> 51

<212> DNA

<213> Artificial Sequence

45 <220>

50 <223> Description of Artificial Sequence:PCR primer

<400> 20

55 cttcgtctct tccccgcgc cattgtcggtt gaagccgttg ttgatgttga a 51

<210> 21

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 21

cttcgtctct tccccgcgc cattgtcagt caggtcgcgg agggtttct c 51

<210> 22

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 22

gacaatggcg cgggggaaga gacgaagtcc 30

<210> 23

<211> 25

<212> DNA



<213> Artificial Sequence

5

<220>

<223> Description of Artificial Sequence:PCR primer

10

<400> 23

15

gccccagaagc ccaaaattga gattt

25

20

<210> 24

<211> 52

<212> DNA

25

<213> Artificial Sequence

<220>

30

<223> Description of Artificial Sequence:PCR primer

<400> 24

35

cttcgtctct tccccgcgc cattgtctgc cgttgccaca ggtgcggcca gc

52

40

<210> 25

<211> 52

45

<212> DNA

<213> Artificial Sequence

50

<220>

<223> Description of Artificial Sequence:PCR primer

55

<400> 25

5 cgcagccagc gatttcatgc gtttcataga ggccaaggct ccttgaatag gt 52

10

<210> 26

<211> 30

15

<212> DNA

<213> Artificial Sequence

20

<220>

<223> Description of Artificial Sequence:PCR primer

25

<400> 26

atgaaacgca tgaaatcgt ggctgcggcg 30

30

<210> 27

35

<211> 25

<212> DNA

<213> Artificial Sequence

40

<220>

<223> Description of Artificial Sequence:PCR primer

45

<400> 27

50

ggatccggag cttatcgact gcacg 25

55

<210> 28

<211> 52

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 28

cgcagccage gatttcatgc gtttcataat tctgtttcct gtgtgaaatt gt 52

<210> 29

<211> 43

<212> PRT

<213> *Corynebacterium glutamicum*

<400> 29

Met Arg Asp Thr Ala Phe Arg Ser Ile Lys Ala Lys Ala Gln Ala Lys

1 5 10 15

Arg Arg Ser Leu Trp Ile Ala Ala Gly Ala Val Pro Thr Ala Ile Ala

20 25 30

Leu Thr Met Ser Leu Ala Pro Met Ala Ser Ala

35 40

<210> 30

<211> 43

<212> PRT

<213> Streptoverticillium mobaraense

<400> 30

Asp Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg

1 5 10 15

Leu Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala

20 25 30

Pro Ala Ala Ser Ser Ala Gly Pro Ser Phe Arg

35 40

<210> 31

<211> 41

<212> PRT

<213> Streptoverticillium mobaraense

<400> 31

Asp Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg

1 5 10 15

Leu Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala

20 25 30

Pro Ala Ala Ser Ser Ala Gly Pro Ser

35 40

5           <210> 32  
             <211> 44  
             <212> PRT  
 10           <213> Streptoverticillium mobaraense

15           <400> 32  
             Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg Leu  
                   1                   5                   10                   15

20           Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala Pro  
                           20                   25                   30

25           Ala Ala Ser Ser Ala Gly Pro Ser Phe Arg Ala Pro  
                           35                   40

30

35           <210> 33  
             <211> 39  
             <212> PRT  
 40           <213> Streptoverticillium mobaraense

45           <400> 33  
             Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg Leu Thr Ala Asp Asp Val  
                   1                   5                   10                   15

50           Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala Pro Ala Ala Ser Ser Ala  
                           20                   25                   30

55

Gly Pro Ser Phe Arg Ala Pro

5

35

10

<210> 34

<211> 45

15

<212> PRT

<213> Artificial Sequence

20

<220>

<223> Description of Artificial Sequence:modified

pro-region of transglutaminase from

25

Streptovercicillium mobaraense

30

<400> 34

Asp Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg

1

5

10

15

35

Leu Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala

20

25

30

40

Pro Ala Ala Ser Ser Ala Gly Pro Ser Phe Gly Pro Lys

35

40

45

45

50

<210> 35

<211> 45

<212> PRT

55

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:modified  
pro-region of transglutaminase from  
Streptovercicillium mobaraense

<400> 35

Asp Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg  
1 5 10 15

Leu Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala  
20 25 30

Pro Ala Ala Ser Ser Ala Gly Pro Ser Phe Gly Pro Arg  
35 40 45

<210> 36

<211> 41

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:modified  
pro-region of transglutaminase from  
Streptovercicillium mobaraense

<400> 36

Asp Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg

1 5 10 15

5

Leu Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala

10

20

25

30

Pro Ala Ala Ser Ser Ala Gly Pro Lys

15

35

40

20

<210> 37

<211> 41

<212> PRT

25

<213> Artificial Sequence

30

<220>

<223> Description of Artificial Sequence:modified

pro-region of transglutaminase from

35

*Streptoverticillium mobaraense*

<400> 37

40

Asp Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg

1

5

10

15

45

Leu Thr Ala Asp Asp Val Ala Asn Ile Asn Ala Leu Asn Glu Ser Ala

20

25

30

50

Pro Ala Ala Ser Ser Ala Gly Pro Arg

35

40

55



<210> 38

<211> 56

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:chimera

pro-region of transglutaminase from  
Streptovercillium mobaraence and  
Streptovercillium cinnamoneum

<400> 38

Asp Asn Gly Ala Gly Glu Glu Thr Lys Ser Tyr Ala Glu Thr Tyr Arg

1

5

10

15

Leu Thr Ala Asp Asp Val Glu Ser Ile Asn Ala Leu Asn Glu Arg Ala

20

25

30

Leu Thr Leu Gly Gln Pro Gly Lys Pro Pro Lys Glu Leu Pro Pro Ser

35

40

45

Ala Ser Ala Pro Ser Arg Ala Pro

50

55

<210> 39

<211> 1079

<212> PRT

<213> Streptomyces albogriseolus

<400> 39

Asn Gly Glu Asn Ser Thr Ala Ala Gly Ser Ser Ala Ser Ala Thr Ala

1 5 10 15

Leu Lys Gly Lys His Arg Val Thr Leu Ile Thr Gly Asp Arg Val Ala

20 25 30

Leu Asp Ala Lys Gly Arg Val Val Gly Leu Glu Pro Ala Glu Gly Arg

35 40 45

Glu His Ile Pro Val Gln Ile Arg Arg Ser Asp Gly His Thr Leu Val

50 55 60

Val Pro Ala Asp Ala Ala Arg Leu Val Ala Ser Gly Lys Leu Asp Gln

65 70 75 80

Arg Leu Phe Asp Val Thr Glu Leu Asn Lys Ala Ala Thr Arg Thr Ala

85 90 95

His Arg Gly Gly Leu Lys Val Ile Val Gly Tyr Arg Gly Ala Ala Lys

100 105 110

Ala Ala Lys Ala Asp Val Arg Asp Ala Gly Thr Val Arg Arg Thr Leu

115 120 125

Thr Ser Leu Asn Ala Asp Ala Val Gln Thr Pro Gln Glu Ala Gly Ala

130 135 140

5           Glu Leu Trp Glu Ala Val Thr Asp Gly Asp Arg Thr Ala Ser Gly Val  
           145                   150                   155                   160

10          Ala Arg Val Trp Leu Asp Gly Val Arg Lys Ala Ser Leu Asp Thr Ser  
                   165                   170                   175

15          Val Gly Gln Ile Gly Thr Pro Lys Ala Trp Glu Ala Gly Tyr Asp Gly  
                   180                   185                   190

20          Lys Gly Val Lys Ile Ala Val Leu Asp Thr Gly Val Asp Ala Thr His  
                   195                   200                   205

25          Pro Asp Leu Lys Gly Gln Val Thr Ala Ser Lys Asn Phe Thr Ser Ala  
                   210                   215                   220

30          Pro Thr Thr Gly Asp Val Val Gly His Gly Thr His Val Ala Ser Ile  
                   225                   230                   235                   240

35          Ala Ala Gly Thr Gly Ala Gln Ser Lys Gly Thr Tyr Lys Gly Val Ala  
                   245                   250                   255

40          Pro Gly Ala Lys Ile Leu Asn Gly Lys Val Leu Asp Asp Ala Gly Phe  
                   260                   265                   270

45          Gly Asp Asp Ser Gly Ile Leu Ala Gly Met Glu Trp Ala Ala Ala Gln  
                   275                   280                   285

50          Gly Ala Asp Ile Val Asn Met Ser Leu Gly Gly Met Asp Thr Pro Glu  
 55

EP 1 219 713 A1

	290	295	300
5			
	Thr Asp Pro Leu Glu Ala Ala Val Asp Lys Leu Ser Ala Glu Lys Gly		
10	305	310	315 320
	Ile Leu Phe Ala Ile Ala Ala Gly Asn Glu Gly Pro Gln Ser Ile Gly		
15		325	330 335
	Ser Pro Gly Ser Ala Asp Ser Ala Leu Thr Val Gly Ala Val Asp Asp		
20		340	345 350
	Lys Asp Lys Leu Ala Asp Phe Ser Ser Thr Gly Pro Arg Leu Gly Asp		
25		355	360 365
	Gly Ala Val Lys Pro Asp Leu Thr Ala Pro Gly Val Asp Ile Thr Ala		
30		370	375 380
	Ala Ser Ala Lys Gly Asn Asp Ile Ala Lys Glu Val Gly Glu Lys Pro		
35		385	390 395 400
	Ala Gly Tyr Met Thr Ile Ser Gly Thr Ser Met Ala Thr Pro His Val		
40		405	410 415
	Ala Gly Ala Ala Ala Leu Leu Lys Gln Gln His Pro Glu Trp Lys Tyr		
45		420	425 430
	Ala Glu Leu Lys Gly Ala Leu Thr Ala Ser Thr Lys Asp Gly Lys Tyr		
50		435	440 445
55			

5	Thr Pro Phe Glu Gln Gly Ser Gly Arg Val Gln Val Asp Lys Ala Ile	450	455	460
10	Thr Gln Thr Val Ile Ala Glu Pro Val Ser Leu Ser Phe Gly Val Gln	465	470	475 480
15	Gln Trp Pro His Ala Asp Asp Lys Pro Val Thr Lys Lys Leu Thr Tyr	485	490	495
20	Arg Asn Leu Gly Thr Glu Asp Val Thr Leu Lys Leu Thr Ser Thr Ala	500	505	510
25	Thr Gly Pro Lys Gly Lys Ala Ala Pro Ala Gly Phe Phe Thr Leu Gly	515	520	525
30	Ala Ser Thr Leu Thr Val Pro Ala Asn Gly Thr Ala Ser Val Asp Val	530	535	540
35	Thr Ala Asp Thr Arg Leu Gly Gly Ala Val Asp Gly Thr Tyr Ser Ala	545	550	555 560
40	Tyr Val Val Ala Thr Gly Ala Gly Gln Ser Val Arg Thr Ala Ala Ala	565	570	575
45	Val Glu Arg Glu Val Glu Ser Tyr Asn Val Thr Leu Lys Val Leu Asp	580	585	590
50	Arg Ser Gly Lys Ala Thr Ala Asn Tyr Met Ala Tyr Leu Ser Gly Leu	595	600	605

5 Thr Gly Leu Gly Lys Asp Arg Ser Tyr Ala Pro Tyr Glu Ala Asp Gly  
 610 615 620

10 Ala Val Ser Val Arg Val Pro Lys Gly Gly Tyr Val Leu Asp Ala Ser  
 625 630 635 640

15 Val Leu Val Gly Ala Asp Pro Glu Thr Trp Arg Gly Ala Asp Trp Leu  
 645 650 655

20 Ala Gln Pro Lys Leu Asp Val Thr Arg Asn Thr Thr Val Thr Val Asp  
 660 665 670

25 Ala Arg Lys Ala Lys Pro Val Lys Val Thr Val Pro Gly Lys Ala Ala  
 675 680 685

30 Lys Ala Gln Phe Ala Ser Ala Asp Tyr Thr Ile Glu Thr Asn Asp Ser  
 690 695 700

35 Ala Val Ser Tyr Gly Trp Trp Leu Glu Asn Tyr Ser Gly Phe Arg Ser  
 705 710 715 720

40 Ala His Leu Gly Pro Gln Ile Thr Asn Gly Thr Leu Ser Gln Gln Trp  
 725 730 735

45 Asn Thr His Phe Ser Asn Gly Ala Lys Ala Gln Tyr Thr Ala Ile Ser  
 740 745 750

50 Gly Gly Lys Val Lys Lys Leu Ala Thr Gly Tyr Thr Arg Ala Phe Lys  
 55

755

760

765

5

Ala Lys Glu Phe Ala Thr Val Gln Val Gly Met Gly Ala Ala Ala Ser

770

775

780

10

Gly Lys Lys Gly Ala Val Thr Ala Phe Gly Trp Leu Pro Gly Ser Ser

785

790

795

800

15

Gly Ala Ser Gly Phe Ser Gln Glu Gln Lys Leu Pro Ser Thr Arg Thr

805

810

815

20

Leu Tyr Leu Ser Thr Val Asn Gly Val Thr Trp Asp Leu Asp Phe Glu

820

825

830

25

Gln Leu Gly Gly Val Asp Asn Glu Gly Trp Pro Ile Tyr Asp Ala Val

835

840

845

30

Tyr Thr Ile Gly Val Gly Lys Thr Tyr Lys Gly Gly Lys Thr Tyr Lys

850

855

860

35

Glu Thr Val Asn Thr Ala Val Phe Gly Pro Arg Leu Thr Ser Ser Tyr

865

870

875

880

40

Gly Val Phe Arg Asp Gly Asn Ser Ile Tyr Gly Val Ile Pro Leu Phe

885

890

895

45

Ala Asp Gly Lys Gly His Ala Gly Ser Ser Glu Phe Ser Ser Ala Val

900

905

910

50

55

5	Thr Thr Leu Tyr Arg Asn Gly Lys Lys Val Gly Ser Asn Asn Asp Pro	915	920	925
10	Leu Phe Gly Glu Glu Gly Phe Thr Val Pro Ser Gly Asp Ala Ala Tyr	930	935	940
15	Arg Leu Thr Thr Ser Val Lys Arg Ser Ala Lys Val Ala Ala Ala Ser	945	950	955 960
20	Thr Arg Ile Asp Ala Ser Trp Thr Phe Arg Ser Lys Lys Thr Ser Gly	965	970	975
25	Glu Lys Gln Leu Pro Val Ser Ser Ala Arg Phe Ala Ala Val Thr Gly	980	985	990
30	Leu Asp Ser Lys Val Ala Ala Gly Lys Lys Ala Thr Phe Pro Val Val	995	1000	1005
35	Val Glu Gly Ala Ala Gln Gly Lys Asn Leu Lys Ser Leu Ala Val Tyr	1010	1015	1020
40	Val Ser Tyr Asn Gly Gly Lys Thr Trp Lys Lys Thr Thr Val Thr Lys	1025	1030	1035 1040
45	Gly Lys Ile Thr Val Lys Asn Pro Ala Lys Gly Lys Ala Ile Ser Phe	1045	1050	1055
50	Arg Ala Lys Ile Thr Asp Lys Lys Gly Asn Ala Ser Leu Ile Thr Ile	1060	1065	1070



His Asn Ala Tyr Tyr Gly Lys

5

1075

10

<210> 40

<211> 444

15

<212> PRT

<213> Streptovercicillium mobaraense

20

<400> 40

Gln Ala Asp Ile Lys Asp Arg Ile Leu Lys Ile Pro Gly Met Lys Phe

1

5

10

15

25

Val Glu Glu Lys Pro Tyr Gln Gly Tyr Arg Tyr Leu Val Met Thr Tyr

20

25

30

30

Arg Gln Pro Val Asp His Arg Asn Pro Gly Lys Gly Thr Phe Glu Gln

35

40

45

35

Arg Phe Thr Leu Leu His Lys Asp Thr Asp Arg Pro Thr Val Phe Phe

50

55

60

40

Thr Ser Gly Tyr Asn Val Ser Thr Asn Pro Ser Arg Ser Glu Pro Thr

65

70

75

80

45

Arg Ile Val Asp Gly Asn Gln Val Ser Met Glu Tyr Arg Phe Phe Thr

85

90

95

50

55

5                   Pro Ser Arg Pro Gln Pro Ala Asp Trp Ser Lys Leu Asp Ile Trp Gln  
                           100                           105                           110

10                   Ala Ala Ser Asp Gln His Arg Leu Tyr Gln Ala Leu Lys Pro Val Tyr  
                           115                           120                           125

15                   Gly Lys Asn Trp Leu Ala Thr Gly Gly Ser Lys Gly Gly Met Thr Ala  
                           130                           135                           140

20                   Thr Tyr Phe Arg Arg Phe Tyr Pro Asn Asp Met Asn Gly Thr Val Ala  
                           145                           150                           155                           160

25                   Tyr Val Ala Pro Asn Asp Val Asn Asp Lys Glu Asp Ser Ala Tyr Asp  
                           165                           170                           175

30                   Lys Phe Phe Gln Asn Val Gly Asp Lys Ala Cys Arg Thr Gln Leu Asn  
                           180                           185                           190

35                   Ser Val Gln Arg Glu Ala Leu Val Arg Arg Asp Glu Ile Val Ala Arg  
                           195                           200                           205

40                   Tyr Glu Lys Trp Ala Lys Glu Asn Gly Lys Thr Phe Lys Val Val Gly  
                           210                           215                           220

45                   Ser Ala Asp Lys Ala Tyr Glu Asn Val Val Leu Asp Leu Val Trp Ser  
                           225                           230                           235                           240

50                   Phe Trp Gln Tyr His Leu Gln Ser Asp Cys Ala Ser Val Pro Ala Thr  
                           245                           250                           255

5           Lys Ala Ser Thr Asp Glu Leu Tyr Lys Phe Ile Asp Asp Ile Ser Gly  
                   260                           265                           270

10          Phe Asp Gly Tyr Thr Asp Gln Gly Leu Glu Arg Phe Thr Pro Tyr Tyr  
                   275                           280                           285

15          Tyr Gln Ala Gly Thr Gln Leu Gly Ala Pro Thr Val Lys Asn Pro His  
                   290                           295                           300

20          Leu Lys Gly Val Leu Arg Tyr Pro Gly Ile Asn Gln Pro Arg Ser Tyr  
           305                           310                           315                           320

25          Val Pro Arg Asp Ile Pro Met Thr Phe Arg Pro Gly Ala Met Ala Asp  
                                   325                           330                           335

30          Val Asp Arg Trp Val Arg Glu Asp Ser Arg Asn Met Leu Phe Val Tyr  
                                   340                           345                           350

35          Gly Gln Asn Asp Pro Trp Ser Gly Glu Pro Phe Arg Leu Gly Lys Gly  
                                   355                           360                           365

40          Ala Ala Ala Arg His Asp Tyr Arg Phe Tyr Ala Pro Gly Gly Asn His  
                                   370                           375                           380

45          Gly Ser Asn Ile Ala Gln Leu Val Ala Asp Glu Arg Ala Lys Ala Thr  
           385                           390                           395                           400

50          Ala Glu Val Leu Lys Trp Ala Gly Val Ala Pro Gln Ala Val Gln Lys  
 55

405

410

415

5

Asp Glu Lys Ala Ala Lys Pro Leu Ala Pro Phe Asp Ala Lys Leu Asp

420

425

430

10

Arg Val Lys Asn Asp Lys Gln Ser Ala Leu Arg Pro

435

440

15

20

<210> 41

<211> 1751

<212> DNA

25

<213> Streptovercillium mobaraense

<220>

30

<221> CDS

<222> (229)..(1659)

35

<400> 41

gctcctatga gcatcgacgc cgccagcagc gatcggttcg gtctgaccgt cgacgccgac 60

40

ggcgagcgcg tgtggctgga cgagcccggc cggcccgtgc cgctcgtgcg gccgtgaaag 120

45

gcccgaaaag agcccaagcc gtgtgaactg cgaggacaaa gggctctggcg caacgcatgt 180

50

caccccagat aagttcgccg cgacctttgc gaaccaggg gagggcgc atg cgc aag 237

Met Arg Lys

1

55

5            get ctc aga tcg ctg ctg gcg gcg tcg atg ctc ata gga gcg atc ggc    285  
           Ala Leu Arg Ser Leu Leu Ala Ala Ser Met Leu Ile Gly Ala Ile Gly  
                  5                            10                            15

10           gcc ggc agc gcc acg gcg gag gcg gcg tcg atc acc gcc ccg cag gcc    333  
           Ala Gly Ser Ala Thr Ala Glu Ala Ala Ser Ile Thr Ala Pro Gln Ala  
 15           20                            25                            30                            35

20           gac atc aag gac cgc atc ctg aag att ccc ggg atg aag ttc gtc gag    381  
           Asp Ile Lys Asp Arg Ile Leu Lys Ile Pro Gly Met Lys Phe Val Glu  
    40                            45                            50

25           gag aag ccc tac cag gcc tac cgc tac ctc gtg atg acg tac cgg cag    429  
           Glu Lys Pro Tyr Gln Gly Tyr Arg Tyr Leu Val Met Thr Tyr Arg Gln  
    55                            60                            65

30           ccg gtg gac cac cgc aat ccc gcc aag ggg acc ttc gag cag cgc ttc    477  
 35           Pro Val Asp His Arg Asn Pro Gly Lys Gly Thr Phe Glu Gln Arg Phe  
    70                            75                            80

40           acc ctg ctc cac aag gac acc gac cgg ccg acc gtg ttc ttc acg tcc    525  
           Thr Leu Leu His Lys Asp Thr Asp Arg Pro Thr Val Phe Phe Thr Ser  
    85                            90                            95

45           ggc tac aac gtc tcc acc aac ccc agc cgc agc gag ccc acg cgc atc    573  
 50           Gly Tyr Asn Val Ser Thr Asn Pro Ser Arg Ser Glu Pro Thr Arg Ile  
           100                            105                            110                            115

55           gtg gac gcc aac cag gtg tcg atg gag tac cgg ttc ttc acg ccg tcc    621

Val Asp Gly Asn Gln Val Ser Met Glu Tyr Arg Phe Phe Thr Pro Ser  
 5 120 125 130

cgg ccg cag ccc gcc gac tgg tcc aag ctg gac atc tgg cag gcg gcg 669  
 10 Arg Pro Gln Pro Ala Asp Trp Ser Lys Leu Asp Ile Trp Gln Ala Ala  
 135 140 145

agt gac cag cac cgc ctg tac cag gcg ctg aag ccg gtc tac ggg aag 717  
 15 Ser Asp Gln His Arg Leu Tyr Gln Ala Leu Lys Pro Val Tyr Gly Lys  
 20 150 155 160

aac tgg ctg gcc acg ggc ggc agc aag ggc ggc atg acg gcc acc tac 765  
 25 Asn Trp Leu Ala Thr Gly Gly Ser Lys Gly Gly Met Thr Ala Thr Tyr  
 165 170 175

ttc cgc cgc ttc tac ccg aac gac atg aac ggc acg gtc gcc tac gtc 813  
 30 Phe Arg Arg Phe Tyr Pro Asn Asp Met Asn Gly Thr Val Ala Tyr Val  
 35 180 185 190 195

gcg ccc aac gac gtg aac gac aag gaa gac tcg gcg tac gac aag ttc 861  
 40 Ala Pro Asn Asp Val Asn Asp Lys Glu Asp Ser Ala Tyr Asp Lys Phe  
 200 205 210

ttc cag aac gtc ggc gac aag gcg tgc cgc acg cag ctc aac tcg gtg 909  
 45 Phe Gln Asn Val Gly Asp Lys Ala Cys Arg Thr Gln Leu Asn Ser Val  
 50 215 220 225

cag cgc gag gcg ctc gtc cgc cgc gac gag atc gtc gcc cgc tac gag 957  
 55 Gln Arg Glu Ala Leu Val Arg Arg Asp Glu Ile Val Ala Arg Tyr Glu

230

235

240

5 aag tgg gct aag gag aac ggc aag acg ttc aag gtc gtc ggc agc gcc 1005  
 Lys Trp Ala Lys Glu Asn Gly Lys Thr Phe Lys Val Val Gly Ser Ala  
 10 245 250 255

15 gac aag gcg tac gag aac gtc gtc ctc gac ctg gtc tgg tcc ttc tgg 1053  
 Asp Lys Ala Tyr Glu Asn Val Val Leu Asp Leu Val Trp Ser Phe Trp  
 260 265 270 275

20 cag tac cac ctg cag agc gac tgc gcc tcc gtc ccc gcc acc aag gcg 1101  
 Gln Tyr His Leu Gln Ser Asp Cys Ala Ser Val Pro Ala Thr Lys Ala  
 25 280 285 290

30 tcc acc gac gag ctg tac aag ttc atc gac gac atc tcg ggc ttc gac 1149  
 Ser Thr Asp Glu Leu Tyr Lys Phe Ile Asp Asp Ile Ser Gly Phe Asp  
 295 300 305

35 ggc tac acc gac cag ggc ctg gag cgc ttc acc ccg tac tac tac cag 1197  
 Gly Tyr Thr Asp Gln Gly Leu Glu Arg Phe Thr Pro Tyr Tyr Tyr Gln  
 40 310 315 320

45 gcg ggc acc cag ctc ggc gcc cct acg gtg aag aac ccg cac ctc aag 1245  
 Ala Gly Thr Gln Leu Gly Ala Pro Thr Val Lys Asn Pro His Leu Lys  
 325 330 335

50 ggc gtg ctg cgg tac ccc ggc atc aac cag ccg cgc tcg tac gtc ccc 1293  
 Gly Val Leu Arg Tyr Pro Gly Ile Asn Gln Pro Arg Ser Tyr Val Pro  
 55 340 345 350 355

5      cgc gac atc ccg atg acc ttc cgc ccc ggc gcg atg gcg gac gtc gac    1341  
       Arg Asp Ile Pro Met Thr Phe Arg Pro Gly Ala Met Ala Asp Val Asp  
                          360                           365                           370

10      cgc tgg gtg cgc gag gac agc cgg aac atg ctc ttc gtg tac ggg cag    1389  
       Arg Trp Val Arg Glu Asp Ser Arg Asn Met Leu Phe Val Tyr Gly Gln  
                          375                           380                           385

15      aac gac ccg tgg agc ggt gaa ccg ttc cgc ctg ggc aag ggc gcc gcc    1437  
 20      Asn Asp Pro Trp Ser Gly Glu Pro Phe Arg Leu Gly Lys Gly Ala Ala  
                          390                           395                           400

25      gcc cgg cac gac tac cgc ttc tac gcc ccg ggc ggc aac cac ggt tcc    1485  
       Ala Arg His Asp Tyr Arg Phe Tyr Ala Pro Gly Gly Asn His Gly Ser  
                          405                           410                           415

30      aac atc gcc cag ttg gtg gcc gac gag cgg gcc aag gcc acg gcc gag    1533  
 35      Asn Ile Ala Gln Leu Val Ala Asp Glu Arg Ala Lys Ala Thr Ala Glu  
       420                           425                           430                           435

40      gtc ctg aag tgg gcc ggt gtg gcg ccg cag gcc gtc cag aag gac gag    1581  
       Val Leu Lys Trp Ala Gly Val Ala Pro Gln Ala Val Gln Lys Asp Glu  
                          440                           445                           450

45      aag gcc gcc aag ccg ctc gcg ccg ttc gac gcc aag ctc gac cgc gtg    1629  
 50      Lys Ala Ala Lys Pro Leu Ala Pro Phe Asp Ala Lys Leu Asp Arg Val  
                          455                           460                           465

55



aag aac gac aag cag agc gcg ctg cgt ccg tagggaccca gtgcgtaagg 1679

Lys. Asn Asp Lys Gln Ser Ala Leu Arg Pro

470

475

cggcgggcgc tcccggcgag gggcgccgc cgtcgcgttc cggaaggccc cgggtgccgc 1739

cgccggtgct tc

1751

<210> 42

<211> 477

<212> PRT

<213> Streptoverticillium mobaraense

<400> 42

Met Arg Lys Ala Leu Arg Ser Leu Leu Ala Ala Ser Met Leu Ile Gly

1

5

10

15

Ala Ile Gly Ala Gly Ser Ala Thr Ala Glu Ala Ala Ser Ile Thr Ala

20

25

30

Pro Gln Ala Asp Ile Lys Asp Arg Ile Leu Lys Ile Pro Gly Met Lys

35

40

45

Phe Val Glu Glu Lys Pro Tyr Gln Gly Tyr Arg Tyr Leu Val Met Thr

50

55

60

Tyr Arg Gln Pro Val Asp His Arg Asn Pro Gly Lys Gly Thr Phe Glu

65

70

75

80

5           Gln Arg Phe Thr Leu Leu His Lys Asp Thr Asp Arg Pro Thr Val Phe  
                                   85                               90                               95

10           Phe Thr Ser Gly Tyr Asn Val Ser Thr Asn Pro Ser Arg Ser Glu Pro  
                                   100                               105                               110

15           Thr Arg Ile Val Asp Gly Asn Gln Val Ser Met Glu Tyr Arg Phe Phe  
                                   115                               120                               125

20           Thr Pro Ser Arg Pro Gln Pro Ala Asp Trp Ser Lys Leu Asp Ile Trp  
                                   130                               135                               140

25           Gln Ala Ala Ser Asp Gln His Arg Leu Tyr Gln Ala Leu Lys Pro Val  
                                   145                               150                               155                               160

30           Tyr Gly Lys Asn Trp Leu Ala Thr Gly Gly Ser Lys Gly Gly Met Thr  
                                   165                               170                               175

35           Ala Thr Tyr Phe Arg Arg Phe Tyr Pro Asn Asp Met Asn Gly Thr Val  
                                   180                               185                               190

40           Ala Tyr Val Ala Pro Asn Asp Val Asn Asp Lys Glu Asp Ser Ala Tyr  
                                   195                               200                               205

45           Asp Lys Phe Phe Gln Asn Val Gly Asp Lys Ala Cys Arg Thr Gln Leu  
                                   210                               215                               220

50           Asn Ser Val Gln Arg Glu Ala Leu Val Arg Arg Asp Glu Ile Val Ala  
                                   225                               230                               235

225                      230                      235                      240

5

Arg Tyr Glu Lys Trp Ala Lys Glu Asn Gly Lys Thr Phe Lys Val Val

245                      250                      255

10

Gly Ser Ala Asp Lys Ala Tyr Glu Asn Val Val Leu Asp Leu Val Trp

260                      265                      270

15

Ser Phe Trp Gln Tyr His Leu Gln Ser Asp Cys Ala Ser Val Pro Ala

20

275                      280                      285

Thr Lys Ala Ser Thr Asp Glu Leu Tyr Lys Phe Ile Asp Asp Ile Ser

25

290                      295                      300

Gly Phe Asp Gly Tyr Thr Asp Gln Gly Leu Glu Arg Phe Thr Pro Tyr

30

305                      310                      315                      320

Tyr Tyr Gln Ala Gly Thr Gln Leu Gly Ala Pro Thr Val Lys Asn Pro

35

325                      330                      335

His Leu Lys Gly Val Leu Arg Tyr Pro Gly Ile Asn Gln Pro Arg Ser

40

340                      345                      350

Tyr Val Pro Arg Asp Ile Pro Met Thr Phe Arg Pro Gly Ala Met Ala

45

355                      360                      365

Asp Val Asp Arg Trp Val Arg Glu Asp Ser Arg Asn Met Leu Phe Val

50

370                      375                      380

55

Tyr Gly Gln Asn Asp Pro Trp Ser Gly Glu Pro Phe Arg Leu Gly Lys  
 385 390 395 400

Gly Ala Ala Ala Arg His Asp Tyr Arg Phe Tyr Ala Pro Gly Gly Asn  
 405 410 415

His Gly Ser Asn Ile Ala Gln Leu Val Ala Asp Glu Arg Ala Lys Ala  
 420 425 430

Thr Ala Glu Val Leu Lys Trp Ala Gly Val Ala Pro Gln Ala Val Gln  
 435 440 445

Lys Asp Glu Lys Ala Ala Lys Pro Leu Ala Pro Phe Asp Ala Lys Leu  
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Asp Arg Val Lys Asn Asp Lys Gln Ser Ala Leu Arg Pro  
 465 470 475

<210> 43

<211> 330

<212> PRT

<213> Streptovercillium cinnamoneum

<400> 43

Ser Asp Asp Arg Glu Thr Pro Pro Ala Glu Pro Leu Asp Arg Met Pro  
 1 5 10 15

5	Glu Ala Tyr Arg Ala Tyr Gly Gly Arg Ala Thr Thr Val Val Asn Asn	20	25	30
10	Tyr Ile Arg Lys Trp Gln Gln Val Tyr Ser His Arg Asp Gly Lys Lys	35	40	45
15	Gln Gln Met Thr Glu Glu Gln Arg Glu Lys Leu Ser Tyr Gly Cys Val	50	55	60
20	Gly Val Thr Trp Val Asn Ser Gly Pro Tyr Pro Thr Asn Arg Leu Ala	65	70	75
25	Phe Ala Ser Phe Asp Glu Asn Lys Tyr Lys Asn Asp Leu Lys Asn Thr	85	90	95
30	Ser Pro Arg Pro Asp Glu Thr Arg Ala Glu Phe Glu Gly Arg Ile Ala	100	105	110
35	Lys Gly Ser Phe Asp Glu Gly Lys Gly Phe Lys Arg Ala Arg Asp Val	115	120	125
40	Ala Ser Val Met Asn Lys Ala Leu Glu Asn Ala His Asp Glu Gly Thr	130	135	140
45	Tyr Ile Asn Asn Leu Lys Thr Glu Leu Thr Asn Asn Asn Asp Ala Leu	145	150	155
50	Leu Arg Glu Asp Ser Arg Ser Asn Phe Tyr Ser Ala Leu Arg Asn Thr	165	170	175

5           Pro Ser Phe Lys Glu Arg Asp Gly Gly Asn Tyr Asp Pro Ser Lys Met  
                   180                   185                   190

10           Lys Ala Val Ile Tyr Ser Lys His Phe Trp Ser Gly Gln Asp Gln Arg  
                   195                   200                   205

15           Gly Ser Ser Asp Lys Arg Lys Tyr Gly Asp Pro Glu Ala Phe Arg Pro  
                   210                   215                   220

20           Asp Gln Gly Thr Gly Leu Val Asp Met Ser Lys Asp Arg Ser Ile Pro  
                   225                   230                   235                   240

25           Arg Ser Pro Ala Lys Pro Gly Glu Gly Trp Val Asn Phe Asp Tyr Gly  
                                   245                   250                   255

30           Trp Phe Gly Ala Gln Thr Glu Ala Asp Ala Asp Lys Thr Thr Trp Thr  
                                   260                   265                   270

35           His Gly Asp His Tyr His Ala Pro Asn Ser Asp Leu Gly Pro Met His  
                   275                   280                   285

40           Val His Glu Ser Lys Phe Arg Lys Trp Ser Ala Gly Tyr Ala Asp Phe  
                   290                   295                   300

45           Asp Arg Gly Ala Tyr Val Ile Thr Phe Ile Pro Lys Ser Trp Asn Thr  
                   305                   310                   315                   320

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325

330

5

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<211> 21

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<210> 45

<211> 32

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32

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<210> 46

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<211> 21

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<400> 46

tacgaattcg agctcggtac c

21

<210> 47

<211> 43

<212> DNA

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43

<210> 48

<211> 46

<212> DNA

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<223> Description of Artificial Sequence:PCR primer



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<211> 26

<212> DNA

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<210> 50

<211> 29

<212> DNA

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:PCR primer

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<210> 51

<211> 30

<212> DNA

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<213> Streptovercillium mobaraense

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1 5 10 15

Val Glu Glu Lys

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<210> 54

<211> 33

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:probe for svPEP

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aagatcccg ggatgaagtt cgtcgaggag aag

33

<210> 55

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:PCR primer

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gaggcggcgt cgatcaccgc ccc

23

5

<210> 56

10

<211> 32

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<213> Artificial Sequence

<220>

20

<223> Description of Artificial Sequence:PCR primer

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32

30

<210> 57

<211> 46

35

<212> DNA

<213> Artificial Sequence

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<220>

<223> Description of Artificial Sequence:PCR primer

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46

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<210> 58

55

<211> 40

<212> DNA

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<223> Description of Artificial Sequence:PCR primer

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40

<210> 59

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:PCR primer

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40

<210> 60

<211> 40

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:PCR primer

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           aatggcgcgg gggaagagac gaagtcctac gccgaaacct           40

10

          <210> 61  
           <211> 40  
 15           <212> DNA  
           <213> Artificial Sequence

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          <220>  
           <223> Description of Artificial Sequence:PCR primer

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          <400> 61  
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35           <210> 62  
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           <223> Description of Artificial Sequence:PCR primer

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55

<210> 63

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40

<210> 64

<211> 40

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<210> 65

<211> 40

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<213> Artificial Sequence

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5 <223> Description of Artificial Sequence:PCR primer

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15 <210> 66

<211> 40

20 <212> DNA

<213> Artificial Sequence

25 <220>

<223> Description of Artificial Sequence:PCR primer

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35 <210> 67

40 <211> 40

<212> DNA

<213> Artificial Sequence

45 <220>

50 <223> Description of Artificial Sequence:PCR primer

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<210> 68

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<221> MOD\_RES

<222> (4)

<223> Xaa is Pro-pNA, pNA is p-nitroanilide

<220>

<223> Description of Artificial Sequence:substrate for  
svPEP

<400> 68

Phe Arg Ala Xaa

1

<210> 69

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:additional  
sequence to native transglutaminase

<400> 69

Ser Ala Gly Pro Lys

1 5

<210> 70

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:additional  
sequence to native transglutaminase

<400> 70

Ser Ala Gly Pro Arg

1 5

# Claims

1. A process for producing a heterologous secretory protein which comprises culturing a coryneform bacterium having an genetic expression construct wherein a nucleic acid sequence encoding a signal peptide domain derived from a coryneform bacterium is connected to the downstream of a promoter sequence which functions in a coryneform bacterium and wherein a nucleic acid sequence encoding a heterologous secretory protein containing a pro-structure part is connected to the downstream of said nucleic acid sequence encoding said signal peptide domain, allowing said coryneform bacterium to produce and secrete said heterologous secretory protein, and then performing the cleavage and elimination of the pro-structure part from said heterologous secretory protein.
2. The method according to claim 1, wherein the heterologous secretory protein is a transglutaminase.
3. The method according to claim 1 or 2, wherein the signal peptide is a signal peptide of a surface protein derived from a coryneform bacterium.
4. The method according to claim 1 or 2, wherein the signal peptide is a signal peptide of a surface protein derived from *Corynebacterium glutamicum*.
5. The method according to claim 4, wherein the signal peptide has the amino acid sequence according to SEQ ID NO: 1 or SEQ ID NO: 29.
6. The method according to claim 1 or 2, wherein the signal peptide is a signal peptide of a surface protein derived

from *Corynebacterium ammoniagenes*.

7. The methods according to claim 6, wherein the signal peptide has the amino acid sequence according to SEQ ID NO: 2.
8. The method according to any one of claims 1 to 7, wherein the pro-structure part corresponds to a pro-structure part of a transglutaminase derived from an actinomycete.
9. The method according to claim 8, wherein the pro-structure part has the sequence according to SEQ ID NO: 3 or SEQ ID NO: 4.
10. The method according to claim 8, wherein the pro-structure part comprises a replacement, deletion, insertion or addition of at least one amino acid, or combination thereof in the amino acid sequence according to SEQ ID NO: 3 or SEQ ID NO: 4.
11. The method according to claim 10, wherein an amino acid sequence of the pro-structure is any one of the amino acid sequences according to SEQ ID NO: 30 to SEQ ID NO: 38.
12. The method according to claim 1 or 2, wherein the cleavage and elimination of the pro-structure part is performed with a protease.
13. The method according to claim 12, wherein the coryneform bacterium which produces and secretes the heterologous secretory protein also produces the protease.
14. The method according to claim 13, wherein the cleavage and elimination of the pro-structure part is performed with the protease and a peptidase.
15. The method according to claim 14, wherein the coryneform bacterium which produces and secretes the heterologous secretory protein further produces the protease and the peptidase.
16. The method according to claim 12 or 14, wherein the protease is derived from an actinomycete.
17. The method according to claim 12 or 14, wherein the protease is derived from *Streptomyces albogriseolus*.
18. The method according to claim 14, wherein the peptidase is derived from an actinomycete.
19. The method according to claim 14, wherein the peptidase is derived from *Streptomyces mobaraense*.
20. The method according to any one of claims 2 to 11, wherein the transglutaminase is a transglutaminase derived from an actinomycete.
21. The method according to claim 20, wherein the transglutaminase is the transglutaminase derived from *Streptovermicillium mobaraense*.
22. The method according to claim 21, wherein the transglutaminase has the amino acid sequence according to SEQ ID NO: 5.
23. The method according to claim 20, wherein the transglutaminase is a transglutaminase derived from *Streptovermicillium cinnamomeum*.
24. The method according to claim 23, wherein the transglutaminase has the amino acid sequence according to SEQ ID NO: 43.
25. A process for secretory production of a mature transglutaminase which comprises producing and secreting a fused protein in a coryneform bacterium, wherein said fused protein is a mature transglutaminase connected to the downstream of a signal peptide derived from a coryneform bacterium.
26. A proline specific peptidase having the following properties:

(1) It reacts on at least one of the following proline containing peptides and cleaves it at the carboxyl terminal side of the proline residue:

Ala-Ala-Pro-pNA, Ala-Phe-Pro-pNA, Phe-Arg-Ala-Pro-pNA

(wherein pNA represents p-nitroanilide)

(2) It has the optimum pH of 6.0-6.5;

(3) It is stable at pH4-9;

(4) It has the optimum temperature of 25-30°C;

(5) It is stable below 20°C;

(6) Its activity is inhibited by phenylmethylsulfonyl fluoride and aminoethylbenzenesulfonyl fluoride hydrochloride;

(7) It has an isoelectric point of 10.2; and

(8) It has a molecular weight of approximately 50,000.

27. The proline specific peptidase according to claim 26, which is derived from an actinomycete.

28. A polypeptide having the proline specific peptidase activity according to claim 26, comprising the amino acid sequence according to SEQ ID NO: 40.

29. The polypeptide having the proline specific peptidase activity according to claim 26, comprising a replacement, deletion, insertion or addition of at least one amino acid, or combination thereof in the amino acid sequence according to SEQ ID NO: 40.

30. A nucleic acid molecule encoding the polypeptide according to claim 28 or 29.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/06780

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int.Cl <sup>7</sup> C12N15/63, C12P21/02, C12N9/10, C12N9/48  According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Int.Cl <sup>7</sup> C12N15/63, C12P21/02, C12N9/10, C12N9/48  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Genbank/EMBL/DDBJ/GeneSeq, WPI (DIALOG), BIOSIS (DIALOG)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 9-316095, A (Mitsubishi Chemical Corporation), 09 December, 1997 (09.12.97) (Family: none)	1-25
Y	JP, 10-108675, A (Ajinomoto Co., Inc.), 28 April, 1998 (28.04.98) (Family: none)	1-25
Y	EP, 198645, A1 (SANKYO CO), 22 October, 1986 (22.10.86) & JP, 6-253838, A & ES, 553735, A & AT, 58556, E & DE, 3675679, C & US, 4985361, A1 & KR, 9403653, B	1-25
Y	Kim, I-G. et al., "The deduced sequence of the novel protransglutaminase E (TGase3) of human and mouse" THE JOURNAL OF BIOLOGICAL CHEMISTRY (1993) Vol.268 No.17 p.12682-12690	2,8-11, 20-25
Y	Duran, R. et al., "Purification, characterisation, and gene clonig of transglutaminase from Streptovercillium cinnamoneum CBS 683.68", Biochimie (1998) Vol.80, No.4 pp.313-319	2,8-11, 20-25
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 18 December, 2000 (18.12.00)		Date of mailing of the international search report 26 December, 2000 (26.12.00)
Name and mailing address of the ISA/ Japanese Patent Office  Facsimile No.		Authorized officer  Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/06780

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, 5-244947, A (Mercian Corporation), 24 September, 1993 (24.09.93) (Family: none)	26-30

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/06780

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

The inventions as set forth in claims 1 to 25 pertain to processes for producing proteins by using coryneform bacteria, while the inventions as set forth in claims 26 to 30 pertain to proline-specific peptidases.

It cannot be recognized that the proline-specific peptidases according to the inventions as set forth in claims 26 to 30 are utilized exclusively for carrying out the inventions as set forth in claims 1 to 25. Such being the case, these two groups of inventions are not considered as relating to a group of inventions so linked as to form a single general inventive concept.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)